THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

EDITED BY

VOLUME XXIV
BALTIMORE
1916

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THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

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STUDIES IN EXPERIMENTAL DIABETES AFTER PANCREATECTOMY.[1]

By ALBERT A. EPSTEIN AND GEORGE BAEHR.

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Frequent estimation of the blood sugar at short intervals has only been possible since the recent introduction of so called micro-chemical methods. Undoubtedly the greatest field of usefulness for these methods is in experimental work on laboratory animals, for they largely eliminate the possibility of a psychic hyperglycemia and of the hyperglycemia following more extensive bleeding. In dogs and cats they also obviate the necessity of obtaining blood from the larger vessels with consequent increase in the blood sugar due to the operative procedures. The advantages gained more than counterbalance the slightly larger limits of technical error that are likely to occur from the use of small quantities of blood.

In the following experiments, a modification of the excellent picric acid method of Lewis and Benedict was used. By means of a technique described recently by one of us,[2] we have been able to obtain remarkably accurate determinations on 0.1 cc. and 0.2 cc. of blood instead of the 2 cc. originally recommended. The method is superior to that of Bang both in simplicity and in accuracy (Epstein[3]).

The frequently repeated estimation of the sugar in such small quantities of blood enabled us to follow the entire course of the hyperglycemia which develops after a pancreatectomy, much more minutely than was previously possible. For this purpose cats were found to be especially suitable, because of the ease with

[1] This work was carried out under the tenure of the George Blumenthal, Jr., and the Moses Heineman Fellowships.

[2] Epstein, A. A., *Jour. Am. Med. Assn.*, 1914, lxiii, 1667.

[3] Epstein, A. A., *Jour. Am. Med. Assn.*, 1915, lxv, 1748.

1

which sufficient blood can be obtained from an ear vein by puncture with a needle, without disturbing the animal.

As originally pointed out by Minkowski,[4] the technique of totally extirpating the pancreas in the cat is more difficult than in the dog, chiefly because of the close relation of one part of the organ to the portal vein. But after a brief experience the ability to do a total extirpation without damage to the portal vein or interference with the blood supply of the duodenum was readily acquired. Post mortem, any tissue near the original site of the organ which even remotely resembled pancreatic tissue was subjected to microscopic examination. In none of the experiments which form the basis of this communication were any fragments of pancreas ever left behind, nor did necrosis of the duodenum ever occur.

In our experience the only disadvantage of using cats is that they succumb sooner than dogs after total pancreatectomy. They are, therefore, less suitable for prolonged metabolic study. But for the purpose of the present investigation, this does not constitute a disadvantage.

After pancreatectomy the animals usually are very thirsty and drink water in large amounts, although they may refuse to eat. This fact necessitated our obtaining some knowledge concerning possible variations in blood volume which might affect the sugar concentration of the blood. For it appeared conceivable that the ingestion of rather large amounts of fluid might cause a dilution of the blood and thus mask an actual increase in its constituents, including sugar. This obviously important point has been universally overlooked by workers in this field. It has been discussed by us in a recent paper dealing with the relation of the percentile to the absolute hyperglycemia.[5]

Changes in the blood volume can be ascertained by means of repeated determinations of the relative amounts of plasma and cells present in the blood. Simultaneously with each sugar determination an estimation of the proportion of plasma and blood cells was therefore always made (by means of a simplified hematocrit) and changes in the blood volume were calculated. It can be safely assumed, for reasons which need not be stated here, that

[4] Minkowski, O., Untersuchungen über den Diabetes mellitus nach Extirpation des Pankreas, Leipsic, 1893, 77.

[5] Epstein, A. A., and Baehr, G., Jour. Biol. Chem., 1914, xviii, 21.

the actual number of cells in the blood stream remains practically constant from hour to hour or even from day to day. A decrease in the proportion of blood cells in a unit of blood therefore signifies an addition to the fluid portion of the blood and hence an increase in the total volume. If the proportion of blood cells found at the beginning of the experiment be taken as the standard for comparison, the subsequent increase or decrease in the blood volume can be ascertained by dividing the percentage of blood cells in a given determination into the percentage found at the outset.

In the following tables Column 2 contains the percentage of plasma, Column 3 the percentage of blood cells, and Column 4 the relative blood volume calculated from the figures in Column 3.

In view of the changes which take place in the blood volume after pancreatectomy, the percentage of sugar found at each determination must therefore be proportionally magnified or decreased, if we are to obtain a correct idea of the actual rate of increase in the sugar content. Column 6 therefore represents the percentage of sugar which would have been found had the blood volume remained as it was before the experiment. This gives us a new and surprising insight into the actual increase in the amount of sugar in the circulating blood.

TABLE I.

Cat 9.

Weight 3,750 Gm.   Complete Pancreatectomy.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	per cent	per cent	per cent	per cent	per cent
Before operation.........	64.3	35.7	100.0	0.084	0.084
After "	65.2	34.8	102.6	0.240	0.266
16......................	72.1	27.9	127.8	0.256	0.327
21......................	69.2	30.8	115.8	0.264	0.306
26.....................	73.5	26.5	134.7	0.320	0.431
42......................	73.4	26.6	134.2	0.320	0.435
52......................	76.3	23.7	150.6	0.280	0.422
70......................	74.7	25.3	141.1	0.290	0.409
96......................	72.1	27.9	127.9	0.372	0.476
100.....................	75.6	24.4	146.3	0.412	0.603
119.....................	73.1	26.9	132.5	0.560	0.742
124.....................	73.4	26.6	134.2	0.580	0.778
141.....................	73.6	26.4	135.2	0.620	0.838

Total glycogen.

	gm.	per cent
Liver	0.184	0.184
Muscle	——	0.224

Incoagulable N in Blood.

Found.	Corrected.
per cent	per cent
0.240	0.324

The blood volume of Cat 9 (Table I) increased after the removal of the pancreas, reaching 134.7 per cent after 24 hours. Subsequently the volume fluctuated above and below this figure, depending on the ingestion of water. The highest value attained by the blood volume was 150.6 per cent 52 hours after the pancreatectomy.

Coincident with the increase in the blood volume the blood sugar content rose gradually from 0.084 per cent before the operation to 0.431 per cent 26 hours after. It then remained at about the same level until 100 hours after the removal of the pancreas when a sudden rise to 0.603 per cent occurred. From that time on, the increase was progressive, reaching 0.838 per cent after 141 hours. The animal which by this time was markedly emaciated became stuporous. The experiment was therefore discontinued; the animal was killed and the liver, muscle, and blood were subjected to further analysis.

This terminal rise in the percentage of blood sugar was observed in three other cats on which similar studies were made after pancreatectomy. (See protocols at end of paper.) In fact the characteristically progressive rise in the blood sugar presaged the death of the animals during the following 24 to 48 hours. This sudden terminal rise appeared, however, at least 24 to 36 hours before the onset of stupor.

As this change in the blood sugar is a terminal phenomenon, one might at first suppose that it represents the result of tissue decomposition with increased sugar production. But the rise always appeared when the animals were still in good condition and quite lively. Furthermore, a simultaneous diminution in the glycosuria demonstrated that the rapidly progressing hyperglycemia was the result of a sugar retention in the blood due to an impaired function of the kidneys.

TABLE II.

Course of Glycosuria in Cat 9.

Time after operation.		Quantity of urine.	Total sugar.		Acetone.	Albumin
hrs.	*hrs.*	*cc.*	*per cent*	*gm.*		
1- 24...	24	125.0	12.5	15.6	0	0
24- 48...	24	252.0	7.3	18.4	+++	0
48- 66...	18	200.0	5.7	11.4	++	+
66- 84...	18	238.0	2.0	4.8	+	+
84-108...	24	202.0	0.6	1.2	0	++
108-141...	33	45.0	+	+	0	++

Table II shows that after 84 hours only traces of sugar appeared in the urine, and that after 48 hours albumin and casts were present in increasing amounts. Coincident with the appearance of the marked albuminuria, the hyperglycemia increased, whereas the glycosuria and acetonuria, which had previously been very marked, progressively diminished. The urine contained no acetone after the 84th hour and only traces of sugar after the 108th hour. In view of the progressively increasing hyperglycemia at this time, the diminution in the excretion of sugar can only be the result of a functional insufficiency of the kidney. Similarly the stupor which began during the last 12 hours of the experiment was probably due to the retention of the acetone bodies, also resulting from the renal insufficiency.

It is interesting to note, however, that the progressive increase in the blood sugar began long before the onset of stupor. As seen in the table, the marked diuresis which began shortly after the operation, persisted for at least 48 hours after the excretion of sugar and acetone began to fail. This fact may be interpreted as an indication that the impairment of function which affected the elimination of sugar and acetone was not of circulatory origin.

The terminal hyperglycemia observed in the cat experiments has its analogue in human diabetes. Whenever exceptionally high sugar values were reported in the literature, we have found that the estimations were made within a day or two of death (Naunyn,[6] 0.7 per cent, Lépine,[7] 1.06 per cent, von Noorden,[8] 0.85 per cent

[6] Naunyn, B., Der Diabetes mellitus. Vienna, 2nd edition, 1906, 189.

[7] Lépine, R., Rev. de méd., 1897, xvii, 832.

[8] Von Noorden, C., Handb. d. Pathologie des Stoffwechsels, Berlin, 2nd edition, 1906, ii, 3.

and 1.01 per cent, etc.). In one case of von Noorden's in which the blood sugar was 1.01 per cent the urine contained only traces of sugar. Post mortem, no nephritis was discoverable. From our experimental observations, it is apparent that all these high figures in human diabetes are probably also due to the retention of sugar resulting from a renal insufficiency. From the experimental evidence it would seem that a progressively increasing percentage of sugar in the blood is an indication of impending death. In a patient with diabetes whom we had the opportunity of observing recently, we found conditions similar to those observed experimentally. In this case as in the experimental animals, a progressive increase in the blood sugar preceded the onset of coma.

In view of the above phenomena it was thought to be of interest to reproduce an absolute renal insufficiency in depancreatized animals, in order to ascertain its effect upon the sugar content of the blood. A total extirpation of the pancreas followed by double nephrectomy was carried out upon six cats, of which three were well nourished and the others had been starved for 9 days.

Table III (Cat 10) is an example of the observation made on the group of well nourished animals.

TABLE III.

Cat 10.

Weight 3,200 Gm. Well Nourished. Pancreatectomy and Double Nephrectomy.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation	64.6	37.4	100.0	0.085	0.085
After "	63.7	36.7	97.2	0.206	0.200
14	75.7	24.3	145.7	0.640	0.932
19	74.7	25.3	140.0	0.816	1.142
24	75.4	24.6	144.0	1.050	1.512
40	77.7	22.3	158.6	1.080	1.713

Total glycogen.

	gm.	*per cent*
Liver	0.278	0.42
Muscle	——	0.99

Incoagulable N in blood.

Found.	Corrected.
per cent	*per cent*
0.280	0.444

Cat 13. Control.

Well nourished. Not operated upon.

Total glycogen.

	gm.	*per cent*
Liver.....................................	11.951	14.40
Muscle	———	1.70

Incoagulable N in blood.

Found.	Corrected.
per cent	*per cent*
0.073	0.073

The figures obtained are very striking. After the operation the blood sugar rose very rapidly. The animal meanwhile exhibited thirst, although it took no food. As both kidneys had been removed, the ingestion of fluid resulted in a simultaneous increase in the blood volume. After 40 hours the blood volume had increased more than 58 per cent. This big factor, as previously stated, must be considered if we are to obtain a correct idea of the actual accumulation of the sugar in the blood. The percentage of sugar found at each determination was therefore proportionately magnified as seen in Column 6. During the course of the experiment the blood sugar thus rose from 0.085 to 1.713 per cent; in other words, it increased more than twentyfold.

TABLE IV.

Cat 20.

Fasted 9 Days before Operation. Initial Weight 3,300 Gm. Final Weight 2,880 Gm. Pancreatectomy and Double Nephrectomy.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	62.3	37.7	100.0	0.058	0.058
After "	58.0	42.0	90.0	0.100	0.090
½......................	56.7	43.3	87.8	0.120	0.104
7......................	65.2	34.8	108.3	0.272	0.295
18......................	70.0	30.0	126.0	0.384	0.484
23......................	74.2	25.8	146.1	0.408	0.596
27......................	74.5	25.5	146.0	0.500	0.730
30......................	76.0	24.0	157.0	0.568	0.892
41......................	76.0	24.0	157.0	0.740	1.161
46......................	77.2	22.8	165.1	0.998	1.647
48......................	77.1	22.9	165.0	1.120	1.980

Total glycogen.

	gm.	per cent
Liver	0	0
Muscle	—	0.06

Incoagulable N in blood.

Found.	Corrected.
per cent	per cent
0.241	0.398

Cat 22.   Control.

Fasted, but not operated upon.

Total glycogen.

	gm.	per cent
Liver	0.014	0.030
Muscle	——	0.560

Incoagulable N in blood.

Found.	Corrected.
per cent	per cent
0.046	0.046

The experiment on Cat 20 (Table IV) serves to illustrate the effect of a similar procedure upon an animal that had fasted for 9 days. In this instance the blood volume gradually increased until at the end of 48 hours it was 65 per cent larger than at the beginning of the experiment. Correcting the sugar values obtained for this dilution, the sugar in the blood is seen to have risen from 0.058 to 1.980 per cent. In other words, at the end of 48 hours the blood contained thirty-four times as much sugar as before the experiment.

The rate of increase in the sugar content of the blood is different in the two sets of experiments presented. In the early stages of the experiment, the hyperglycemia developed much more rapidly in the well nourished than in the starved animals. The difference is best seen by comparing the curves of the results obtained on Cat 10, which was well nourished, with that of Cat 20, which was starved.

The blood sugar of Cat 10 is as high after 19 hours as that of Cat 20 after 40 hours. The very rapid increase in the hyperglycemia in Cat 10 continued for the first 24 hours, but proceeded rather slowly after that. The progress of the hyperglycemia in Cat 20, on the other hand, was much slower than in Cat 10 for

the first 41 hours; after this the rise in the sugar content was very rapid.

The difference in the rate of increase in the hyperglycemia in the two sets of animals can be accounted for by a difference in the reserve of carbohydrate. A control to Cat 10 (Cat 13) which was not operated upon, showed 14.4 per cent of glycogen in the liver and 1.7 per cent in the muscles. (Glycogen was determined
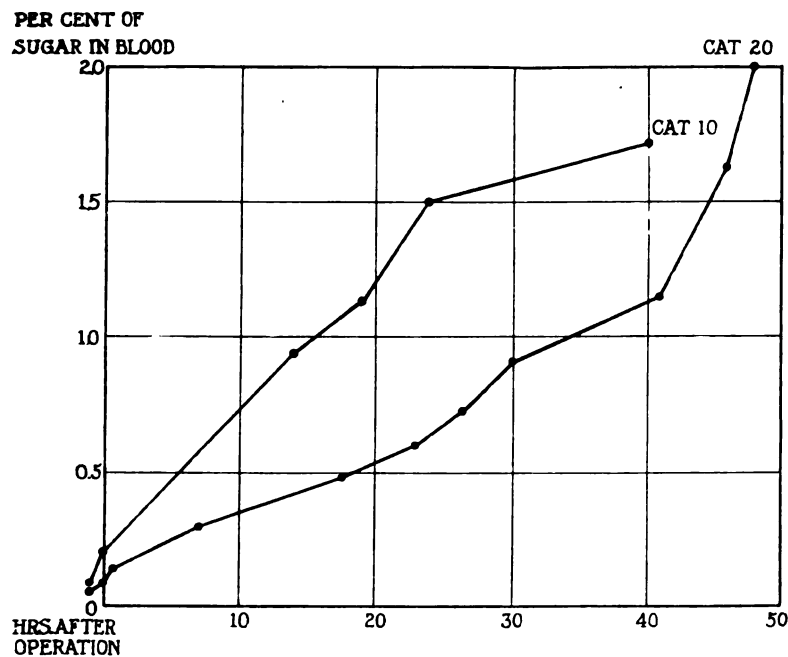
Fig. 1. Curves illustrating rate of increase in blood sugar after pancreatectomy and double nephrectomy.

Cat 10 well nourished; Cat 20 fasted.

by Pflüger's method.) The liver of the control animal to Cat 20 (Cat 22), which had also been starved for 9 days, but had not been operated upon, contained only 0.03 per cent of glycogen, and the muscles 0.56 per cent. On the other hand, the livers and muscles of Cats 10 and 20 yielded the following results:

Cat 10.	Cat 20.
Liver.......0.42 per cent glycogen.	Liver....... 0 per cent glycogen.
Muscle......0.99 " "	Muscle........0.06 " "

Evidently the extraordinary accumulation of sugar in the blood does not hinder the progressive decomposition of glycogen. In both animals the accumulation of sugar in the blood can, at least in part, be accounted for by a mobilization of the reserve carbohydrate from the liver and muscles. That this is true is further evidenced by the much more rapid accumulation of sugar in the blood of the well nourished animal (Cat 10) in which the carbohydrate reserve before the operation was very much greater. That the glycogenolysis is gradual and not sudden may be implied from the gradual rise in the blood sugar and from the fact that some glycogen is still present in the organs at the end of the experiments.

The incoagulable nitrogen in the blood of these animals was also determined (Folin's method), but the results do not permit any deductions concerning the existence of a dextrose-nitrogen ration in the blood. It is possible that similar experiments on animals rendered totally glycogen-free, by means of starvation and adrenalin, may yield more definite knowledge as to the derivation of blood sugar from tissue protein. One point concerning the incoagulable nitrogen deserves mention, and that is the fact that the actual amount of this substance in the blood is found much greater when a correction is made for change in the blood volume.

CONCLUSIONS.

1. In experimental diabetes after pancreatectomy marked changes in blood volume occur.

2. It is essential to allow for such changes in blood volume in order to obtain an accurate estimation of the variations in the sugar, incoagulable nitrogen, and other constituents of the blood.

3. The hyperglycemia in experimental diabetes after pancreatectomy in the cat mounts progressively in the terminal stages of the condition because of diminution in the permeability of the kidneys.

4. This furnishes an explanation of the excessive rise in the blood sugar of diabetics prior to the development of coma.

5. Experimental interference (by double nephrectomy) with the glycosuria of depancreatized animals also causes a rapidly progressing hyperglycemia.

6. This accumulation of sugar in the blood is largely due to the gradual mobilization of carbohydrate from the liver and muscles.

TABLE V.
Cat 2.
Weight 2,720 Gm.   Pancreatectomy on Normal Animal.

Time.	Blood sugar.	
hrs.	*per cent*	
Before operation.............	0.079	
After "	0.266	
15...........................	0.250	
24...........................	0.292	Relative blood
36...........................	0.212	volume not de-
44...........................	0.238	termined in
60...........................	0.208	this animal.
72...........................	0.246	
84...........................	0.244	
108..........................	0.456	
111½.........................	0.512 (killed.)	

	Weight. *gm.*	Glycogen. *gm.* *per cent*	
Liver................................ 74		0.213	0.3
Muscle............................... 44		0.326	0.74

TABLE VI.
Cat 3.
Weight 3,190 Gm.   Pancreatectomy on Normal Animal.

Time.	Blood sugar.	
hrs.	*per cent*	
Before operation....................	0.090	
After "	0.272	Relative blood
12...............................	0.286	volume not de-
36...............................	0.272	termined in
64...............................	0.290	this animal.
89...............................	0.380	
100..............................	Died.	

TABLE VII.
Cat 32.
Weight 1,900 Gm.   Pancreatectomy.   Starved 10 Days.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	56.3	43.7	100.0	0.058	0.058
After "	58.1	41.9	104.3	0.106	0.110
43......................	61.8	38.2	114.4	0.193	0.221

Animal died subsequently; no further analyses were made.

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TABLE VIII.

Cat 31.

Weight 2,300 Gm.   Pancreatectomy.   Starved 10 Days.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	55.2	44.8	100.0	0.055	0.055
After "	48.6	51.4	81.1	0 107	0 093
20......................	54.1	45.9	97.9	0.226	0.221
86......................	50.6	49.4	90.7	0.760	0.689

TABLE IX.

Cat 5.

Weight 2,400 Gm.   Nephrectomy on Normal Cat.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	63.5 *	36.5	100.0	0.088	0.088
After "	59.1	40.9	89.2	0.254	0.227
14......................	63.9	36.1	101.1	0.140	0.142
24......................	64.0 .	36.0	101.4	0.150	0.152
40......................	68.5	31.5	115.9	0.122	0.141
48......................	68.2	31.8	114.8	0.109	0.125
63......................	68.0	32.0	114 1	0.140	0.160
88......................	65.2	34.8	101.9	0.148	0 155
88½......................	Died.				

TABLE X.

Cat 6.

Weight, 2,800 Gm.   Nephrectomy on Normal Animal.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	68.1	31.9	100.0	0.079	0.079
After "	70.4	29.6	107.9	0.308	0.332
18......................	71.9	28.1	113 5	0.120	0.136
43......................	78.8	21.2	150.5	0.180	0 271

TABLE XI.

Cat 8.

Nephrectomy on Normal Animal.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	57.9	42.1	100.0	0.079	0.079
After "	60.0	40.0	105.2	0.360	0.379
17.........................	72.7	27.3	154.2	0.140	0.216
24.........................	73.0	27.0	155.9	0.142	0.221

TABLE XII.

Cat 21.

*Weight 3,100 Gm. Nephrectomy. Starvation and Phlorhizin. Starved
5 Days. 2 Days Phlorhizin (0.5 Gm.). Weight before Operation
2,680 Gm.; at Death 2,550 Gm.*

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	60.9	39.1	100.0	0.042	0.042
After "	50.0	50.0	78.2	0.077	0.060
6.........................	Not taken.			0.196	
16.........................	64.0	36.0	108.6	0.080	0.087
21¾.........................	60.6	39.4	99.2	0.070	0.069
24½.........................	61.1	38.9	100.5	0.076	0.076
29.........................	61.9	38.1	102.6	0.078	0.080
40.........................	62.8	37.2	105.1	0.090	0.095
45.........................	62.0	38.0	102.9	0.117	0.120
47.........................	61.1	38.9	100.5	0.125	0.126

Killed.

	Weight.	Glycogen.
	gm.	*gm.*
Liver.............................	100	3.717
Muscle......................................	'87.5	0.152
Incoagulable N in blood....... ... .0.281 gm. per 100 cc.		

TABLE XIII.

Cat 24.

Nephrectomy. Starved 9 Days. Phlorhizin Twice (0.5 Gm.).

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	44.4	55.6	100.0	0.057	0.057
After operation (5 min.).	61.0	39.0	142.6	0.102	0.145
6......................	39.4	60.6	91.7	0.081	0.074
20......................	44.7	55.3	100.5	0.085	0.085
25......................	43.5	56.5	98.8	0.075	0.074
30......................	50.9	49.1	113.2	0.060	0.068

Died during next 6 hours.

TABLE XIV.

Cat 26.

Nephrectomy. Starvation and Phlorhizin. Fasted 9 Days. Phlorhizin Twice (0.5 Gm.). Weight before Killing 2,300 Gm.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	61.4	38.6	100.0	0.052	0.052
After "	57.0	43.0	89.8	0.078	0.070
13......................	61.7	38.3	100.8	0.080	0.081
23......................	59.6?	40.4	95.5	0.064	0.061

Killed.

	Weight. *gm.*	Glycogen. *gm.*
Liver...	81	1.362
Muscle...	74	0.055

Incoagulable N in blood (15 cc.)..............0.136 gm. per 100 cc.

TABLE XV.

Cat 12.

Weight 2,830 Gm. Nephrectomy and Pancreatectomy on Normal Animal.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	64.6	35.4	100.0	0.090	0.090
After "	57.05	42.95	82.3	0.210	0.173
17½....................	73.2	26.8	132.0	0.480	0.633
21¾....................	75.6	24.4	145.1	0.720	1.044
22½....................	77.0	23.0	154.0	0.930	1.432

TABLE XVI.

Cat 4.

Nephrectomy and Pancreatectomy on Normal Animal.

Time.	Blood sugar.	
	per cent	
Before operation...................	0.089	
After onset of anesthesia...........	0.099	Relative blood
10 min. after pancreatectomy.......	0.206	volume not de-
40 " " "	0.224	termined in this
(10 min. after nephrectomy.)		animal.
8½ hours after pancreatectomy.....	0.392	
16 " " "	0.540	
20¼ " " "	0.700	
25 " " "	0.900	

TABLE XVII.

Cat 23.

Weight 2,200 Gm.   *Pancreatectomy and Nephrectomy.*   *Fasted 9 Days before Operation.   Phlorhizin.*

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	57.5	42.5	100.0	0.050	0.050
During "	53.0	47.0	90.4	0.062	0.056
After "	54.2	45.8	93.0	0.095	0.088
6½......................	59.2	40.8	104.1	0.210	0.219
20......................	68.5	32.5	130.8	0.618	0.808
24½......................	73.1	26.9	158.0	0.680	1.074
30..........'...........	73.7	26.3	161.6	0.690	1.115

Cat died subsequently; other analyses not made.

TABLE XVIII.

Cat 25.

Weight 2,860 Gm.   *Pancreatectomy and Nephrectomy.*   *Fasted 10 Days.*
Phlorhizin (0.5) Gm. on 5th and 6th Days.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Before operation.........	48.7	51.3	100.0	0.050	0.050
After "	39.5	60.5	84.8	0.064	0.054
14......................	51.0	49.0	104.7	0.260	0.272
21......................	57.0	43.0	119.3	0.456	0.544
23......................	58.8	41.2	124.5	0.416	0.518

	Weight. gm.	Glycogen. gm.
Liver.. 90		0.014
Muscle....................................... 82		0 074
Incoagulable N in blood...............0.145 gm. per 100 cc.		
Corrected...........................0.180 " " 100 "		

TABLE XIX.

Cat 29.

Initial Weight, 3,950 Gm.; Final, 2,920 Gm.   Nephrectomy.   Starvation and Phlorhizin.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	per cent	per cent	per cent	per cent	per cent
Before operation.........	48.2	51.8	100.0	0.059	0.059
After "	43.3	56.7	91.4	0.130	0.119
43......................	48.4	51.6	100.5	0.088	0.088

Killed.

	Weight. gm.	Glycogen. gm.
Liver.. 72		0.748
Muscle....................................... 65		0.126
Incoagulable N in blood..................0.183 gm. per 100 cc.		

TABLE XX.

Cat 30.

Initial Weight, 3,500 Gm.; Final, 2,100 Gm.

Time.	Blood.		Relative blood volume.	Blood sugar.	
	Plasma.	Cells.		Found.	Corrected.
hrs.	per cent	per cent	per cent	per cent	per cent
Before operation.........	54.5	45.5	100.0	0.057	0.057
After "	53.3	46.7	97.4	0.154	0.150
43......................	58.5	41.5	109.6	0.072	0.079

	Weight. gm.	Glycogen. gm.
Liver.. 95.5		2 196
Muscle...:................................... 62.5		0.167
Incoagulable N in blood... .... 0 284 gm. per 100 cc.		

THE EFFECT OF PHLORHIZIN ON THE FORMATION OF GLYCOGEN IN THE LIVER.[1]

By ALBERT A. EPSTEIN and GEORGE BAEHR.

(*From the Pathological Laboratory of the Mount Sinai Hospital, New York.*)

Although it is now generally recognized that the glycosuria of phlorhizin poisoning is due to a direct action on the kidney, some authors (Grube,[2] Biedl and Kolisch,[3] Gigon[4]) still believe that phlorhizin may also possess a specific effect upon the liver. The following observations are presented because they conclusively controvert this possibility of a direct mobilization of liver glycogen by phlorhizin. In fact they seem to indicate an interesting condition which has not previously been surmised, that in the absence of the kidneys the presence of phlorhizin in the body actually stimulates the accumulation of glycogen in the liver.

Many of the following experiments were carried out for another purpose and formed the basis of the previous communication upon some "Studies in Experimental Diabetes after Pancreatectomy." They may be classified into seven series as seen in the following tables. In all the experiments cats weighing 2,500 to 3,000 gm. were used. Phlorhizin was administered subcutaneously, 0.5 gm. in 5 cc. of olive oil, on 2 successive days.

The glycogen was determined by Pflüger's method. The blood sugar was determined by means of the microchemical method described by one of the authors.[5]

The experiments tabulated in Tables I, II, and III demonstrate the result of starvation and of starvation plus phlorhizin upon the

[1] This work was carried out under the tenure of the George Blumenthal, Jr., and the Moses Heineman Fellowships.

[2] Grube, K., *Arch. f. d. ges. Physiol.*, 1909, cxxviii, 118.

[3] Biedl, A., and Kolisch, R., *Verhandl. d. Cong. f. inn. Med.*, 1900, xviii, 573.

[4] Gigon, A., *Ztschr. f. klin. Med.*, 1907, lxiii, 420.

[5] Epstein, A. A., *Jour. Am. Med. Assn.*, 1914, lxiii, 1667.

glycogen content of the liver and muscles. The most marked effect is exerted upon the liver, the glycogen in five of the seven animals being reduced to little more than a trace, and completely disappearing in two others that received phlorhizin.

The experiments in Table V seem to indicate that bilateral nephrectomy also results in a mobilization of carbohydrate from the liver and to a less extent from the muscles. This is evidenced by the disappearance of most of the glycogen from the

TABLE I.

Normal Animal.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
83	11.951	14.4	88	1.460	1.66	0.078

TABLE II.

Starved for 5 to 9 Days.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
40	0.008	0.02	55.0	0.063	0.11	0.079
47	0.014	0.03	33.5	0.188	0.56	
45.1	0.025	0.06	41.3	0.021	0.05	
31.0	0.013	0.04	32.0	0.005	0.02	
Average		0.04			0.19	

TABLE III.

Starved and Phlorhizinized for 9 Days.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
68	0.204	0.30	53.5	0.148	0.28	0.065
98			74.0	0.170	0.23	0.064
48.5			47.5	0.118	0.25	0.047
Average					0.25	

TABLE IV.

Starved and Phlorhizinized for 9 Days before Operation. Then Double Nephrectomy. Starved 3 More Days and Killed.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar.	
						Before operation.	Before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
100	3.717	3.72	87.5	0.152	0.17	0.042	
81	1.362	1.68	74.0	0.055	0.07	0.052	
72	0.748	1.04	65.0	0.126	0.20	0.059	0.088
95.5	2.196	2.29	62.5	0.167	0.27	0.057	0.079
Average		2.18			0.18		

TABLE V.

Animals 3 Days after Double Nephrectomy. Starved during the 3 Days. Well Fed before Operation.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar.	
						Before operation.	Before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*
61	0.880	1.28				0.088	0.155
75	0.278	0.37				0.079	0.150
75	0.044	0.05	81	0.593	0.73	0.079	0.156
Average		0.57					

TABLE VI.

Pancreatectomy and Double Nephrectomy. Well Fed before Operation. Killed 2 Days after Operation.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
66	0.278	0.42	85	0.845	0.99	0.085
114	0.750	0.66	90	2.850	3.17	0.090
Average		0.54				

TABLE VII.

Starved and Phlorhizinized for 9 Days. Pancreatectomy and Double Nephrectomy. Killed 2 Days after Operation.

Weight of liver.	Glycogen.		Muscle.	Glycogen.		Blood sugar before killing.
gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*	*per cent*
79			103.5	0.064	0.06	0.058
90	0.014	0.02	82.0	0.074	0.09	0.050
Average					0.07	

liver of cats which were previously well fed and by the hyperglycemia which was present after the operation.

Pancreatectomy and double nephrectomy upon previously well fed cats resulted in a rapid diminution though not a complete disappearance of the liver glycogen in 48 hours (Table VI). 48 hours after a similar operation upon cats which were previously phlorhizinized and starved for 9 days (Table VII) the liver was found to be practically glycogen-free.

Important and interesting results were obtained in the four experiments listed in Table IV. These animals were phlorhizinized and starved for 9 days and then a double nephrectomy was performed. After 3 more days of starvation they were killed. Before the operation there was a fairly marked hypoglycemia because of the long continued phlorhizin glycosuria. After the nephrectomy the percentage of blood sugar rose to the normal figure. The analyses of the muscles demonstrated the presence of glycogen in amounts similar to that found in animals starved, or starved and phlorhizinized for a similar length of time but in which the kidneys had not been removed (Tables II and III).

The results of the analyses of the livers (Table IV) were, however, very striking. From the results obtained in the seven experiments of Tables II and III it was obvious that at the time of the operation the livers of all four animals must have contained little or no glycogen. And yet 3 days subsequent to the operation considerable quantities of glycogen were present in the liver although the animals had continued to starve. Starvation and phlorhizin for this length of time ordinarily free the liver of its glycogen (Tables II and III). The operation of double

nephrectomy in a normal animal also induces a rapid diminution of the liver glycogen.

There are only two possible explanations to account for the reaccumulation of glycogen in the liver of phlorhizinized animals after nephrectomy. One is that because of a possible disturbance in carbohydrate metabolism produced by the phlorhizin, an excess of sugar accumulates in the body after the nephrectomy. This explanation is rendered improbable by the fact that the percentage of blood sugar in these animals never rose above the normal figure.

The only possible explanation therefore appears to be that phlorhizin exerts a specific effect upon the liver which does not become evident unless the kidneys are removed. In other words, phlorhizin in some way stimulates the liver to build up glycogen. With the kidneys intact, the glycosuric action of phlorhizin is so much more powerful than this liver action that the glycogen accumulations in the liver are rapidly diminished. Upon removal of the kidneys, however, the glycosuric action is eliminated and the effect upon the liver becomes manifest in a rapid building up of glycogen, even in a state of extreme starvation.

THE USE OF PHLORHIZINIZED DOGS TO DETERMINE THE UTILIZABLE CARBOHYDRATE IN FOODS.

THE FOOD VALUE OF COMMERCIAL GLUCOSE.

By W. D. SANSUM and R. T. WOODYATT.

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The sum total of *d*-glucose which a given food may introduce into the metabolism will depend (1) upon its content of free glucose and of polysaccharides that yield glucose by digestion; and (2) upon its content of other substances which, with or without digestion, are capable of transformation into *d*-glucose within the body; for example, other sugars besides glucose, polysaccharides whose digestion yields other sugars besides glucose, and proteins and protein digestion products. While in many cases ordinary methods of proximate food analysis may enable us to estimate the glucose equivalents of foods with sufficient accuracy for clinical purposes, this is not true for all foods. The soluble fraction of the nitrogen-free extract may contain a mixture of sugars incapable of separation or exact measurement, the insoluble portion may not be sharply separable into the utilizable and nonutilizable fractions, etc.

The idea suggested itself of passing a food through the body of a completely phlorhizinized dog and measuring its total yield of extra sugar in the urine, the well known phlorhizin technique being thus applied to a problem of food analysis. The extra sugar recovered would then naturally represent nothing which was either indigestible, unabsorbable, or unassimilable. If the food contained protein, such sugar as came from this source could be reckoned from the urinary nitrogen and would not be confused with extra sugar.

On previous occasions, for purposes of ascertaining directly their value in diabetic management, we have determined the

total glucose equivalents of breads by metabolism experiments with completely phlorhizinized dogs and obtained results which were in close harmony with the values calculated from the proximate food analysis. The present paper describes an attempt to utilize the same method to determine directly what .percentage of commercial glucose, when fed by mouth, is capable of ultimate absorption and assimilation as d-glucose. Five experiments were performed, in each of which the dogs received a weighed dose of commercial glucose by mouth, and a control dose of a corresponding weight of chemically pure d-glucose. The results are given in Table I.

TABLE I.

Results of the Administration of Commercial Glucose (Corn Syrup) and d-Glucose to Phlorhizinized Dogs.

Experiment No.	Weight of administered substance.		Weight of extra urinary sugar.		Given substance recovered as d-glucose.		Remarks.
	Chemically pure d-glucose (Kahlbaum).	Water-free commercial glucose.	After d-glucose.	After commercial glucose.	d-Glucose.	Commercial glucose.	
	gm.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	
1......	15	16	10.99	12.19	73.27	76.19	Oral administration.
2......	8	8	4.19	4.01	52.38	50.13	" "
3......	16	16	12.06	11.87	75.38	74.19	" "
4......	16	16	13.80	12.57	86.25	78.56	d-Glucose subcutaneously; commercial glucose by mouth.
5......	16	16	11.97	10.69	74.81	66.81	Oral administration.
Total.	71	72	53.01	51.33	74.66	71.29	" "

DISCUSSION.

It will be noted that in all five experiments there were given 71 gm. of pure d-glucose, of which 53.01 gm., or 74.7 per cent, reappeared in the urine as extra sugar. The highest percentage of any single dose recovered was 86.25 per cent in Experiment 3, the lowest 52.38 per cent in Experiment 2. In other words, the control doses of d-glucose did not reappear quantitatively in the

urine. We have previously obtained results of this sort following doses of substances which, according to the literature, should reappear quantitatively in the urine. A number of possibilities might be suggested in explanation: 1. Some of the glucose might be destroyed in the bowel by bacteria; accordingly, in Experiment 4 the material was given subcutaneously. The yield of extra sugar was higher in this case but still not quantitative. 2. The completeness of the phlorhizination might be questioned, but this possibility we have repeatedly tested and eliminated. 3. Some glucose might be retained in the body and burned. This process might be favored if the rate at which sugar entered the body exceeded that at which it was eliminated, since in such a contingency the concentration of sugar in the tissues (including the blood) would tend to rise and favor oxidation. We have found that too concentrated and rapidly given doses of sugar are as a matter of fact less likely to be quantitatively excreted than slowly given dilute doses, also that it is easier to obtain quantitative returns from starch than from sugar. These observations favor the view that some sugar is retained or burned in the body. We have also observed that more nearly quantitative results were formerly obtained at a time when we had been using Lusk's older method of giving phlorhizin dissolved in carbonate solution at 6 hour intervals, instead of by the Coolen method as at present. In the older method the dogs thus received alkali which was omitted in the later work. We have accordingly tried maintaining a free diuresis through administration of salines supplemented by suitable quantities of alkali during the experiment, and in this way the best yields have been obtained. This seems to suggest that such failures to obtain quantitative returns as those demonstrated in this series may be due to relative impairments of the elimination rate, caused by the effects of acid on the kidneys and other tissues. However this may be, the value of the present series of experiments as throwing light on the utilizability of commercial glucose is maintained by the parallelism between the experiments with this substance and the d-glucose controls.

A comparison of the returns from the pure glucose with those obtained from equivalent weights of commercial glucose for all five experiments shows that they stand in the ratio of 74.6 per cent for the pure substance to 71.29 per cent for the commercial

glucose, the dry substance in the latter yielding therefore in all 95.48 per cent as much extra sugar as the same weight of pure d-glucose. On the basis that the sample of commercial glucose submitted for study contains 20 per cent of water, 100 gm. of the solid residue is capable of affecting the urinary output of d-glucose from completely phlorhizinized and glycogen-free dogs in the same manner as though it contained 95.48 per cent of pure d-glucose. This implies that at least 95.48 per cent of the solid matter in the sample of commercial glucose studied is capable of passing through whatever physiological processes of digestion, absorption, and assimilation are necessary for its ultimate existence in the body as d-glucose.

The animals showed no evidences of any deleterious effects attributable to the ingestion of commercial glucose, notwithstanding the large size of the doses (1 to 2 gm. per kilo of body weight in 3 to 6 hours), and the great sensitiveness of completely diabetic animals to toxic influences.

The experiments are considered of interest as showing the possibility of using the diabetic organism for resolving a heterogeneous mixture of carbohydrates and allied bodies into the form of a single easily measured sugar, and as casting light on the composition and utilizability of that mixture of glucose, dextrins, and other substances known as commercial glucose.

EXPERIMENTAL PART.

Animals.—Healthy adult female dogs which had borne pups and which weighed 10 to 16 kg. were used exclusively. They were kept in metabolism cages with water but no food.

Collection of Urine.—The urine was drawn by catheter and the bladder flushed out with warm water at 6 hour intervals, the process ending with the withdrawal of the catheter after the flushing and sharply at the end of each period. Additional catheterizations were performed when the animals showed a tendency to urinate in the cage between the regular 6 hour catheterization periods. The urine for each period was collected in a liter volumetric flask and made up to the mark with distilled water.

Drugs.—The phlorhizin used was Merck's. The epinephrin was Parke, Davis and Company's adrenalin in ampules, of which a fresh one was used for each dose.

Phlorhizination.—1 gm. of phlorhizin was triturated with 20 cc. of olive oil and injected subcutaneously at the beginning of the experiment and once every 24 hours thereafter throughout the experiment.

Deglycogenation.—Epinephrin, 0.04 mg. per kilo of body weight, subcutaneously, was given once every 3 hours until G:N was constant. As soon as a constant output of glucose and nitrogen and a constant G:N were attained, the epinephrin was discontinued in accordance with a method described by us in a previous paper.[1]

Analytical Methods.—Glucose in the urine was determined by the titration method of Bang and Bohmannson and by the polariscope, at intervals also both before and after fermentation of the urine with ordinary yeast. Nitrogen was determined according to Kjeldahl.

Commercial Glucose.—The material used was an almost clear, colorless, heavy syrup known as commercial glucose or corn syrup, obtained in the open market. Of this a 10 per cent solution was made which showed in the polariscope a reading of $[10] + 11.95°$. By titration according to Bang's method the original material had a reducing power corresponding to a 36.5 per cent solution of *d*-glucose. It contained 20 per cent of water.

For administration, the material was weighed into 10 or 20 gm. portions, diluted with 10 volumes of water, and given by mouth in several small fractions at $\frac{1}{2}$ to 1 hour intervals. 20 gm. of the original syrup have been reckoned as 16 gm. of water-free substance, on the basis of a 20 per cent H_2O content.

Chemically Pure d-Glucose.—A white, crystalline Kahlbaum's "Traubenzucker" preparation was used. The substance after pulverization and drying was weighed out, made up to 8 per cent strength with water, and the resulting solution checked by titration and the polariscope. The dose of *d*-glucose was then meas-

[1] Sansum, W. D., and Woodyatt, R. T., Studies on the Theory of Diabetes. V. Narcotic Drugs in Phlorhizin Diabetes, *Jour. Biol. Chem.*, 1915, xxi, 1.

ured with a volumetric pipette and administered in the same manner as the corn syrup.

Tabular Record of Experiments.—These show the course of the experiments by 6 and 12 hour periods of time. The term "extra glucose" is always reckoned as $G - (N \times G{:}N)$, in which G represents the grams of urinary glucose for the period in question; N, the grams of urinary nitrogen; and $G{:}N$, the quotient obtained by dividing the grams of glucose for the fore-periods by the grams of nitrogen for the same periods.

EXPERIMENT 1.

Period.	Length of period.	Glu-cose.	Nitro-gen.	G:N.	Extra glu-cose.	Remarks.
	hrs.	*gm.*	*gm.*		*gm.*	
1....	24	Preparation period.				
2....	6	15.66	3.36	4.66		
3....	6	14.14	4.66	3.03		
4....	6	11.62	4.16	2.79		
5....	6	12.66	4.70	2.70		
6....	6	*19.31*	*3.49*	*5.53* ⎱		
					12.19	20 gm. commercial glucose.
7....	6	11.85	3.42	3.46 ⎰		
8....	6	*16.88*	*2.63*	*6.42* ⎱		
					10.99	15 gm. Kahlbaum's glucose.
9....	6	10.00	3.16	3.15 ⎰		
10....	6	10.05	3.39	2.96		

EXPERIMENT 2.

1....	24	Preparation period.				
2....	6	5.55	1.99	2.79		
3....	6	5.44	1.96	2.87		
4....	6	*8.24*	*1.58*	*5.22* ⎱		
					4.01	10 gm. commercial glucose.
5....	6	5.09	1.76	2.89 ⎰		
6....	6	4.80	1.93	2.49		
7....	6	*8.07*	*1.51*	*5.34* ⎱		
					4.19	8 gm. Kahlbaum's glucose.
8....	6	4.91	1.64	2.99 ⎰		
9....	6	4.91	1.81	2.71		
10....	6	5.26	1.85	2.84		

EXPERIMENT 3.

Period.	Length of period.	Glu-cose.	Nitro-gen.	G:N.	Extra glu-cose.	Remarks.
	hrs.	*gm.*	*gm.*		*gm.*	
1....	24	Preparation period.				
2....	6	8.23	3.23	2.55		
3....	6	7.40	3.04	2.43		
4....	6	*17.23*	*2.53*	*3.81*		
					11.87	20 gm. commercial glucose.
5....	6	5.85	1.97	2.97		
6....	6	*16.29*	*2.00*	*8.14*		
					12.06	16 gm. Kahlbaum's glucose.
7....	6	5.71	1.99	2.86		
8....	6	5.86	2.31	2.51		

EXPERIMENT 4.

Period.	Length of period.	Glu-cose.	Nitro-gen.	G:N.	Extra glu-cose.	Remarks.
1....	24	Preparation period.				
2....	6	14.25	2.69	5.30		
3....	6	10.28	3.89	2.64		
4....	6	10.43	4.09	2.55		
5....	6	10.43	4.21	2.48		
6....	6	*20.01*	*3.37*	*5.94*		
					12.57	20 gm. commercial glucose.
7....	6	9.60	3.39	2.83		
8....	6	*21.34*	*3.42*	*6.24*		
					13.80	16 gm. Kahlbaum's glucose given
9....	6	8.31	2.87	2.88		subcutaneously.
10....	6	7.28	2.81	2.59		

EXPERIMENT 5.

Period.	Length of period.	Glu-cose.	Nitro-gen.	G:N.	Extra glu-cose.	Remarks.
1....	72	Preparation period.				No epinephrin used.
2....	6	10.06	2.55	3.94		
3....	6	8.58	2.07	4.14		
4....	12	*26.53*	*3.92*	6.77	10.69	20 gm. commercial glucose.
5....	12	*24.54*	*3.11*	7.89	11.97	16 gm. Kahlbaum's glucose.
6....	12	18.04	4.14	4.35		
7....	12	15.47	4.40	3.73		

CAN CARBON DIOXIDE IN SEA WATER BE DIRECTLY DETERMINED BY TITRATION?[1]

BY SERGIUS MORGULIS AND EVERETT W. FULLER.

(From the United States Fisheries Biological Station, Woods Hole.)

In two recent papers[2] Moore and his collaborators have arrived at startling conclusions regarding the metabolism of marine organisms. The method pursued by Moore and his coworkers in experiments with a variety of sea animals was extremely simple. The animals were kept in closed vessels of different capacity (2.8 to 45 liters), and the content of carbon dioxide and oxygen of the sea water was determined in aliquot portions at the beginning and end of each experiment. The oxygen was measured by the method of Winkler. The carbon dioxide, on the other hand, was determined by a method of titrating the sea water with $\frac{N}{100}$ acid or alkali, using phenolphthalein as indicator.

It is to be regretted that nowhere in his papers does Moore discuss this method of carbon dioxide determination, for the introduction of which he is responsible, and no information is given beyond the statement that the titrations were made with $\frac{N}{100}$ solutions, employing four drops of the indicator for each 100 cc. of the sea water.

With data thus obtained Moore computes the respiratory quotient, or ratio between the produced carbon dioxide and consumed oxygen, which has gained such importance in all metabolism studies. A review of the results recorded in the papers referred to brings out two significant points: first, the wide fluctuations of the quotients in consecutive experiments; and, second, their high values. We can demonstrate this by the random choice of a series of respiratory quotients obtained in experiments with an unidentified fish during 12 hour periods. The quotients were:

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[1] Published by permission of the Commissioner of Fisheries.

[2] Moore, B., Edie, E. S., Whitley, E., and Dakin, W. J., The Nutrition and Metabolism of Marine Animals in Relationship to (a) Dissolved Organic Matter and (b) Particulate Organic Matter of Sea Water, *Biochem. Jour.*, 1911-12, vi, 255. Moore, Edie, and Whitley, The Nutrition and Metabolism of Marine Organisms; the Rate of Oxidation and Output of Carbon Dioxide in Marine Animals in Relation to the Available Supply of Food in Sea Water, *Report on the Lancashire Sea-Fisheries*, Liverpool, 1913, xxii, 297.

1st day	1.38 – 0.98	6th day	1.56 – 0.96
2nd "	1.17 – 1.13	7th "	2.00 – 1.12
3rd "	0.87 – 1.00	8th "	2.00 – 1.30
4th "	1.28 – 1.09	9th "	2.76 – 1.91
5th "	0.99 – 0.95	10th "	1.90 – 0.95

The respiratory quotient which reflects the nature of the combustion in the organism varies under physiological conditions between 0.70 and 1.00, depending upon the relative amounts of fat, protein, or carbohydrate oxidized. Respiratory quotients which lie outside these limits are not necessarily abnormal and usually indicate synthetic processes accompanying the metabolic activity of the organism. The range of variation, however, is more or less narrow, and is occasioned by determinable factors.

The quotients of the above example have an extraordinary range of variation from 0.95 to 2.76. This is more surprising since the fish was fasting and presumably under uniform conditions throughout the experiment. The respiratory quotient in inanition apparently always tends to a low level corresponding to the combustion of protein and fat upon which the organism subsists; this was also found to hold true in the case of fasting fish.[3]

Each of the facts pointed out above would be sufficient reason for questioning the accuracy of Moore's observations. It is, however, his conception of the metabolism of aquatic animals, which he would isolate from the rest of the animal world, that concerns us. Moore considers that he has demonstrated by his researches that high respiratory quotients (even indeterminable ones) are the expression of inherent peculiarities of their metabolism, resulting from changes in the intramolecular complex. To further substantiate his view, Moore directs attention to Vernon's valuable investigation of the gaseous metabolism of lower aquatic organisms, who also records high respiratory quotients. The allusion to Vernon's results is, however, ill chosen, as he observed such quotients on rare occasions and only when the animals were asphyxiated.

We believe that the erroneous conclusion was drawn from undoubtedly inaccurate facts, and that both must therefore be traced to the method of carbon dioxide determination in the sea water. The method of measuring the carbon dioxide by the changes in acidity of the water, though sound in principle, is encumbered with serious complications in a system as complex as that represented by sea water. And it must always be borne in mind that the CO_2 may exist not only in a dissociated condition as HCO_3, but also as a gas in solution. Only the former would be determined by titration.

The difficulty of getting a sharp and unmistakable end-point when titrating with $\frac{N}{100}$ solutions and phenolphthalein is alone

[3] Morgulis, S., Studies on Fasting Flounders, *Jour. Biol. Chem.*, 1915, xx, 37.

sufficient to condemn the method where great accuracy is essential. In a large series of titrations of sea water we have become convinced that many factors, such as the amount of indicator, length of time since the indicator has been added, excessive agitation of the water—all may cause appreciable differences in the titration. Even under conditions as uniform as it was possible to secure, the titration values were subject to considerable fluctuations. From a large number of determinations we obtained the following average results:

Sea water. cc.	$\frac{N}{100}$ Acid. cc.
100	1.57
200	3.94
400	6.95
Ratio............ 1:2:4	1:2.5:4.4

These average results, however, do not show the extent of the experimental error. This can be better judged from the fact that the maximum and minimum titration values deviate 10 to 15 per cent from the average.

By the nature of the experiments, where animals are kept in unaerated water, the amount of carbon dioxide which may be allowed to accumulate is small, since the supply of dissolved oxygen can last them only for a limited period. Unless the analysis can be made with great accuracy, as the samples employed for titration must necessarily be a small fraction of the total volume, the error will be greatly multiplied in computation.

Leaving out of consideration these purely technical objections we shall turn our attention to the value of the method *per se.* This we attempted to determine in two ways: first, by checking up the results of the titration against those obtained by a method which we will describe presently; secondly, by determining the per cent of known quantities of carbon dioxide added to water, which we were able to recover by either method.

The method which we utilized for checking the results of titration consisted in precipitating the CO_2 in the water with a standard solution of $Ba(OH)_2$ and measuring the excess by titration. With sea water $Ba(OH)_2$ gives a very bulky precipitate which consists, in addition to the barium carbonate, of barium sulphate and probably also of magnesium and calcium hydroxide. But the amount required to saturate completely a given

volume of sea water is easily found out, and by repeated trials extending over several weeks we became convinced that this amount is constant. With this as a basis we added a reasonable excess of the standard $\frac{N}{5}$ Ba(OH)$_2$ to accurately measured samples of water and titrated aliquot portions of the clear supernatant liquid with $\frac{N}{10}$ or $\frac{N}{50}$ acid. The results obtained by this method are still far from being entirely satisfactory, but the method is susceptible of development and we intend to experiment further with it.

Determinations carried out on samples of the same water by the method of direct titration and by the precipitation method gave invariably much higher results by the latter. To study the titration method more effectively we analyzed waters to which known quantities of carbon dioxide had been added. For lack of better means, we did it by weighing a flask with a measured quantity of water, before and after passing a current of carbon dioxide through it. The carbon dioxide was made from calcium carbonate and chemically pure hydrochloric acid. The gas was passed through sulphuric acid, then through the sample of water. A few minutes were enough to increase appreciably the weight of the flask. The weighings were made on a balance sensitive to the third decimal. After the water was weighed the analyses were made as quickly as possible. From the results of these analyses, given in the subjoined table, the per cent of carbon dioxide recovered was computed.

Water.	CO$_2$ added.	CO$_2$ recovered by titration.	
	gm.	*gm.*	*per cent*
Sea water	0.060	0.0257	43.0
	0.130	0.0275	21.2
	0.169	0.0365	21.6
	0.058	0.0172	30.0
Tap water	0.47	0.1530	32.6
	0.73	0.2450	33.6
	0.06	0.0207	34.5
	0.18	0.0564	31.3
	0.26	0.1181	45.4
	0.11	0.0593	54.0
Distilled water	0.022	0.0040	18.0
	0.051	0.0104	20.3

We may conclude from these data that on the average not more than 33 per cent of the carbon dioxide can be recovered by titration. In that case, provided the oxygen determinations were correct, the respiratory quotients ought to have been abnormally low. We cannot reconcile our findings concerning the inefficiency of the method with the high respiratory quotients which Moore obtained by it. In a series of experiments which we made with several species of fish (minnow, cunner, scup, mackerel) in which we measured the oxygen consumption by Winkler's method and the carbon dioxide by titrating with $\frac{N}{100}$ alkali, as Moore did, we found respiratory quotients varying from 0.19 to 1.14, the low quotients being by far more predominant. But no results obtained by a method of such questionable value are of importance, nor can general conclusions derived from them receive serious consideration.

THE STABILITY OF THE GROWTH-PROMOTING SUBSTANCE IN BUTTER FAT.[1]

By THOMAS B. OSBORNE AND LAFAYETTE B. MENDEL.

WITH THE COOPERATION OF EDNA L. FERRY AND ALFRED J. WAKEMAN.

(From the Laboratory of the Connecticut Agricultural Experiment Station and the Sheffield Laboratory of Physiological Chemistry in Yale University, New Haven.)

A considerable number of observations are now on record to show that certain mixtures of isolated food substances furnishing a ration upon which animals (albino rats) decline or cease to grow can be converted by the addition of some of the natural "fats" into a ration adequate for growth.[2] We have found that the inefficiency of lard and some other fats in this respect is not due to the destruction of the growth-promoting factor by heat, since these fats fail to promote growth adequately even when they are prepared at low temperatures in the laboratory.[3] Furthermore we found that butter fat does not lose its growth-promoting potency by treatment with live steam;[4] and the yolk extracts of heated eggs have also been reported as effective.[5] From such

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[2] McCollum, E. V., and Davis, M., *Jour. Biol. Chem.*, 1913, xv, 167. Osborne, T. B., and Mendel, L. B., *Jour. Biol. Chem.*, 1913, xv, 311; 1913-14, xvi, 423. McCollum and Davis, *Proc. Soc. Exper. Biol. and Med.*, 1914, xi, 101. Osborne and Mendel, *Jour. Biol. Chem.*, 1914, xvii, 401. McCollum and Davis, *Jour. Biol. Chem.*, 1914, xix, 245. Osborne and Mendel, 1915, xx, 379; *Proc. Soc. Exper. Biol. and Med.*, 1915, xii, 92. McCollum and Davis, *Jour. Biol. Chem.*, 1915, xx, 641; xxi, 179. See also Wheeler, R., *Jour. Exper. Zool.*, 1913, xv, 209. MacArthur, C. G., and Luckett, C. L., *Jour. Biol. Chem.*, 1915, xx, 161.

[3] Osborne and Mendel, *Jour. Biol. Chem.*, 1914, xvii, 401; 1915, xx, 380.

[4] Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xx, 381.

[5] McCollum and Davis, *Proc. Soc. Exper. Biol. and Med.*, 1914, xi, 101

facts it seems unlikely that the explanation of the ultimate fail-
ure of growth when certain of the natural fats supply the fat com-
ponent of the diet is to be found in some destructive reaction
brought about by preliminary heating. McCollum and Davis[6]
believe that the substance which exerts a stimulating action on
the growth of rats is sufficiently stable to withstand conditions
of saponification which they have employed.

We have found[7] that by fractional crystallization from alcohol
it is possible to concentrate the growth-promoting factor present
in butter fat and beef fat. It remains in the mother liquor or
"oil" fractions, whereas the fractions containing the fats with
high melting points are ineffective. In order to learn whether
the growth-promoting substance retains its physiological potency
when kept for long periods, samples of butter fat and butter "oil,"
prepared in accordance with published directions,[7] were stored
under different conditions. Their efficiency in restoring growth,
etc., was subsequently tested on animals that had failed on the
"lard diets." It seemed not unlikely that after remaining a long
time in the light at room temperatures changes in the physiologi-
cally active component of natural fats and oils might ensue such
as would render them less potent than before in relation to growth.
The feature here involved has a practical bearing in relation to
the storage of butter and other fat products.

A quantity of butter "oil" was prepared on September 25, 1914,
from part of a lot of butter fat which had previously been found
efficient in promoting growth according to our usual experimental
procedure. Samples of the butter fat as well as the butter "oil"
were preserved:

> No. 1 at 18° C., in the light (18° L't).
> No. 2 at 18° C., in the dark (18° D'k).
> No. 3 at 8° C., in the dark (8° D'k).

The diets of the rats were composed of:

	per cent
Casein	18
Starch	24
"Protein-free milk"	28
Lard	30

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[6] McCollum and Davis, *Jour. Biol. Chem.*, 1914, xix, 245.

[7] Osborne and Mendel, *Jour. Biol. Chem.*, 1915, xx, 379.

A part of the lard was replaced by the butter fat or butter "oil" in the experimental recovery period.

The outcome with the *butter "oil"* was as follows: The sample 18° L't still showed potency in November, 1914. A cessation of growth was stopped, continuance of growth was attained, and the attending sore eyes[8] were healed, in Rat 2414 ♀. In March, 1915, the same sample occasioned only transitory improvement and did not stop the early cessation of growth and decline of body weight in Rat 2508 ♂. Butter fat produced a satisfactory recovery. In July, 1915, failure to restore growth was recorded (Rat 2804 ♀). The "sore eyes" were not healed. Here again fresh butter fat produced a good response.

Butter "oil" 8° D'k was still potent in effecting restoration of growth and healing the "sore eyes," in December, 1914 (Rat 2422 ♀). In August, 1915, this potency was no longer manifest (Rat 2921 ♂); but the animal was restored by addition of butter fat 8° D'k to the diet.

With the *butter fat* samples the results were different. All of them, 18° L't, 18° D'k, and 8° D'k, are still efficient, like fresh butter fat, at the present time (December, 1915), more than a year after their preparation.

The foregoing experience, to which little would be added by graphic presentation of the curves of body weight, indicates the pronounced stability of the growth-promoting substance as contained in butter fat under ordinary conditions of storage. However, in the butter "oil," in which the growth-promoting factor is more concentrated than in the original fat, gradual deterioration occurred, so that within a year this characteristic potency was eventually almost completely lost.

[8] Cf. Osborne and Mendel, *Jour. Biol. Chem.*, 1913–14, xvi, 431.

CEPHALIN.

II. BRAIN CEPHALIN.[1]

By P. A. LEVENE and C. J. WEST.

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In recent years a number of important contributions to the chemistry of cephalin have appeared, particularly those by Parnas and his coworkers.[2] Through the efforts of these workers the principal components have been identified as cephalinic acid ($C_{18}H_{33}O_2$), stearic acid, aminoethyl alcohol, glycerol, and phosphoric acid. Assuming that the molecule of cephalin is composed of equimolecular proportions of these components, we may expect the following composition of the substance:

$$C_{41}H_{78}NPO_8, \text{ with C} = 66.17; \text{ H} = 10.57; \text{ N} = 1.88; \text{ P} = 4.17$$

There is, however, a considerable discrepancy between these theoretical values and those found by most observers. The discrepancy, of course, could be explained by the fact that the physical properties of cephalin are of a nature which does not permit the preparation of a sample in sufficient purity. This view, however, is not tenable in the light of our experience with lecithin. Lecithin, both in physical and chemical properties, resembles cephalin closely, and yet the empirical analytical values obtained on the substance by various observers always agree with the theory.

On the other hand, if the empirical composition is correct then the present information regarding the nature of the components of cephalin has to be regarded as incomplete.

In view of this the work on cephalin was resumed, principally from the two following standpoints: (1) Is the composition of ceph-

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913-14, xvi, 419.

[2] Parnas, J., *Biochem. Z.*, 1909, xxii, 411. Baumann, A., *ibid.*, 1913, liv, 30. Renall, M. H., *ibid.*, 1913, lv, 296.

Author.	Source.	C	H	N	P	NH₂	Me
Thudichum[3]...............	Brain	60.00	9.38	1.68	4.27		
Zuelzer[4].................	Ox brain	60.20	9.80	3.80	2.60		
Koch[5]...................	Sheep brain	59.50	9.80	1.75	3.85		1.73
Cousin[6].................	Brain			1.82	3.89		
Stern and Thierfelder[7]...	Egg yolk	59.68	9.74	1.57	3.64		
Falk[8]...................	{ Nerves	55.75	9.66	1.94	4.42		
	{ Human brain	57.56	9.21	2.93	3.23		
Neubauer[9]..............	Brain	{ 61.99	9.85	1.65	3.44		
		{ 62.12	9.87	1.69	3.45		
Parnas[10]................	Brain			1.83	3.86		
Frank[11].................	Liver	57.10	9.62	1.72	3.91		
Baumann[12]..............	Human brain			1.84		1.63	
Renall[13]................	{ Ox brain			1.69	3.56	1.37	
	{ Sheep brain			1.92	4.27	1.60	
Bürger and Beumer[14]....	Erythrocytes			1.78	4.06		

alin constant? (2) Do the quantities of the components obtained on hydrolysis agree with those required by the theory, if the prevailing hypothesis of the structure of cephalin is correct?

By varying the nature of the organic solvents and of the temperature at which precipitation or extraction took place we could not change the composition of the resulting phosphatide. Attempts were then made to purify the substance through the lead salt. The analysis of the lead salt gave values for cephalin identical with those of the original substance. When the free cephalin was liberated from the lead salt it again manifested a composition identical with that of the original material. Finally,

[3] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884, 57.

[4] Zuelzer, G., Z. f. physiol. Chem., 1899, xxvii, 262.

[5] Koch, W., Z. f. physiol. Chem., 1902, xxxvi, 136.

[6] Cousin, H., Compt. rend. Soc. de biol., 1906, lxi, 23.

[7] Stern, M., and Thierfelder, H., Z. f. physiol. Chem., 1907, liii, 381.

[8] Falk, F., Biochem. Z., 1908, xiii, 163; 1909, xvi, 190.

[9] Fränkel, S., and Neubauer, E., Biochem. Z., 1909, xxi, 321.

[10] Parnas, Biochem. Z., 1909, xxii, 411.

[11] Frank, A., Biochem. Z., 1913, l, 273.

[12] Baumann, Biochem. Z., 1913, liv, 30.

[13] Renall, M. H., Biochem. Z., 1913, lv, 296.

[14] Bürger, M., and Beumer, H., Biochem. Z., 1913, lvi, 450.

the free phosphatide obtained through the lead salt was reconverted into the lead salt, and this again did not alter the composition of the resulting substance. On the basis of this, one is inclined to conclude that so called cephalin contains some other substance besides the enumerated components. The results of the quantitative hydrolysis of a purified sample showed that the sum of the found components averaged only 90 per cent of the original weight of the substance.

There is nothing to force the conclusion that the substance unaccounted for is an integral part of the molecule. It may be an impurity. We, therefore, concluded to make an attempt to convert cephalin into a saturated phosphatide by reduction with hydrogen after Paal. These experiments were under way before the appearance of Paal's work on the hydrogenation of lecithin.[15] Unfortunately the behavior of the substance towards the reducing agent is very disappointing.

The reduction of cephalin proceeds at a very slow rate, so that for the present we are in possession of only small quantities of material.

The samples thus far analyzed gave analytical values analogous to those of the original material. However, the experience on sphingomyelin has shown that often thirty recrystallizations are required before the substance is obtained in a sufficient degree of purity. Hence we hope to reach a conclusion regarding the true composition of cephalin through further purification of the hydro derivative. Work towards that end is in progress.

EXPERIMENTAL PART.

Preparation of Cephalin.

The cephalin-lecithin fraction of the brain, representing the ether-soluble portion of the alcoholic extract, which still contained some of the cerebroside fraction (cerebrin and sphingomyelin) and some cholesterol, was first purified by precipitating a concentrated ether or petroleum ether solution with dry acetone and then repeatedly with 95 per cent alcohol. After the second or third precipitation from alcohol the precipitate contained little

[15] Paal, C., and Oehme, H., *Ber. d. chem. Ges.*, 1913, xlvi, 1297.

or no ether-insoluble material (cerebroside fraction). The composition of the cephalin did not change after the fifth or sixth precipitation. This material was then either twice suspended in absolute acetone and stirred vigorously for about 2 hours to completely dehydrate it, or better, precipitated two or three times from a concentrated ether solution with dry acetone. Under these conditions the cephalin precipitated as a slightly yellow granular powder, which gradually darkened upon drying, forming a light or dark brown powder. This was easily pulverized. While the moist cephalin is hygroscopic the dried product is stable and easily handled. Prepared in this way cephalin contains a varying amount of ash.

An ash-free preparation may be obtained as follows: The cephalin is emulsified with water by grinding it with a small amount of water in a mortar to a fine paste and then adding water, with stirring, until a thin emulsion results. This is then precipitated by adding 10 per cent hydrochloric acid until no further precipitate forms. The cephalin separates as a light yellow amorphous mass which filters slowly with suction. It is best separated from the water by centrifuging the mixture. The moist product is taken up in dry ether and the solution either dried with sodium sulphate, concentrated, and precipitated, or better, concentrated at once and precipitated with dry acetone.

The same object is more rapidly accomplished by decanting off most of the dilute acid, filtering the cephalin over cheese-cloth, and extracting the cephalin with ether. The difficulty of extracting with ether, in that it forms a gelatinous emulsion,[16] is overcome by the addition of absolute alcohol, care being taken that the amount added does not precipitate the cephalin.

In either case repeated precipitation is necessary to obtain an anhydrous product. This method is accompanied by considerable loss of material, because of the solubility of the cephalin in the acidulated water.

The same object is obtained, probably with less loss of material, by shaking an ether solution of cephalin with 10 per cent hydrochloric acid solution several times, according to Parnas,[17] then

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[16] Thudichum, *loc. cit.*, 58.

[17] Parnas, *loc. cit.*

with water, and precipitating as above. The emulsions which may result are best prevented by the addition of absolute alcohol. Such a sample gave the following figures upon analysis:

0.1542 gm. substance gave 0.3420 gm. CO_2 and 0.1312 gm. H_2O.
0.1516   "   "   "   0.3342 "   "   " 0.1250 "   "
0.5000   "   "   neutralized 7.0 cc. $\frac{N}{10}$ HCl.
0.2000   "   "   in acetic acid, gave 6.4 cc. N gas at 20° and 750 mm.
0.3000   "   "   gave 0.0410 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{78}NPO_8$:	Calculated[18] for $C_{41}H_{78}NPO_{13}$:	Found:
C	66.17	60.00	60.49
			60.13
H	10.57	9.55	9.52
			9.22
N	1.88	1.70	1.96
NH$_2$ N	1.88	1.70	1.79
P	4.17	3.76	3.80

The formula $C_{41}H_{78}NPO_8$ is based on the assumption that the molecule contains 1 molecule each of aminoethyl alcohol, $H_2NCH_2CH_2OH$, glycerophosphoric acid, $C_3H_5(OH)_2OPO(OH)_2$, stearic acid, $C_{18}H_{36}O_2$, and cephalinic acid, $C_{18}H_{32}O_2$. As is seen from the analytical figures, and as has already been mentioned in the introduction, there is a marked discrepancy between these figures and the calculated ones. The formula $C_{41}H_{78}NPO_{13}$ agrees much better with the analytical figures found.

Attempts to Purify Cephalin.

Thinking that perhaps our methods of purification were not sufficiently rigorous, we submitted our material to the following treatments.

A portion of cephalin was dissolved in boiling amyl alcohol (50 gm. require about 250 cc. for solution) and the solution quickly cooled. The cephalin separated as a thick, amorphous mass. This was ground up with dry acetone and repeatedly washed with the same solvent.

A second lot was dissolved in hot ethyl acetate (10 gm. require about 200 cc. for solution). Upon cooling the solution in the ice box the cephalin precipitated out nearly quantitatively.

[18] Fränkel and Neubauer, *Biochem. Z.*, 1909, xxi, 321.

This was washed with dry acetone as above. The composition of the cephalin in either case remained unchanged, so that there was no purification by these methods. This is in agreement with the observations of Baskoff[19] on liver heparphosphatide.

We then examined the method used successfully by MacLean[20] in obtaining cuorin. This consists in extracting the crude material with alcohol at 60–70° for several hours and repeating the process several times. Finding that this operation did not give us any purification, we modified the method as follows. The cephalin was dissolved in a small amount of gasoline, boiling at 50–60°, or in ether, and poured into 98 per cent alcohol which was kept at about 60° during the operation. A part of the cephalin settles out as a thick oil on the bottom and sides of the flask and on the stirrer, while a part remains in the alcohol-gasoline or the alcohol-ether mixture. The precipitate is taken up in ether and precipitated with acetone. The solution is concentrated in vacuum, the residue taken up in ether and precipitated. Since the two lots of material have the same composition no fractionation was effected. The following figures were obtained upon analysis of the fraction which remained in the alcohol-ether mixture:

0.1482 gm. substance gave 0.3260 gm. CO_2 and 0.1274 gm. H_2O.
0.5000 "   "   neutralized 6.33 cc. $\frac{N}{10}$ HCl.
0.3000 "   "   gave 0.0388 gm. $Mg_2P_2O_7$.

	C	H	N	P
Found:	60.00	9.62	1.78	3.60

Properties of Cephalin.

There is little to add to the many properties already given by Thudichum, Falk, Parnas, and Fränkel. It may be well to call attention to certain differences which have been noted in the literature. Thudichum states that cephalin is soluble in anhydrous ether, while Parnas[17] says it is not. We have found that perfectly anhydrous ether does not dissolve cephalin, but remains clear for some time. A drop of water added to the ether at once produces the characteristic deep red, fluorescent solution.

[19] Baskoff, A., Z. f. physiol. Chem., 1908, lvii, 395.

[20] MacLean, H., Z. f. physiol. Chem., 1908, lvii, 304; Biochem. Jour., 1909, iv, 168; 1912, vi, 333.

The above mentioned method of purification shows that cephalin is markedly soluble in warm 98 per cent alcohol (it does not precipitate out when the ether is removed). Koch and Falk state that it is insoluble in cold or hot alcohol. This may be influenced by the fact that the cephalin is added as an ether solution; the same concentration cannot be reached by boiling cephalin with alcohol. That cephalin is somewhat soluble in cold alcohol is easily seen in the loss on repeated precipitation.

The precipitation of cephalin from an aqueous emulsion or suspension by hydrochloric acid is probably a process of coagulation and does not depend upon the formation of a hydrochloride.[21] Fränkel and Neubauer[18] express the same view and show that the same precipitation is brought about by organic acids and mineral salts.

Cephalin may be obtained in suspension as a nearly colorless substance by precipitation with hydrochloric acid. All attempts to dry it in such a way as to retain this color have failed. As soon as the water is decanted off and acetone is added, the brown color appears. If ether is used, this brown color appears instantly. The color seems to have no influence upon the composition of the molecule.

Lead Compound of Cephalin.

Since the various methods of fractional precipitation and extraction of cephalin failed to give us a product that corresponded to the one theoretically expected, we attempted a purification by chemical means. The most promising method seemed to be through the lead compound. Such a derivative of cephalin (peroxycephalin) has been described by Thudichum.[22] He prepared it by treating an ether solution of cephalin with a warm alcoholic solution of lead acetate and purified it by precipitating from ether with alcohol.

The lead compound of cephalin was prepared as follows: 20 gm. of cephalin were dissolved in 200 cc. of boiling amyl alcohol and this hot solution was treated with a boiling solution of about 25 gm. of lead acetate in amyl alcohol. The deep red solution was then allowed to cool nearly to room temperature and an ex-

[21] Thudichum, loc. cit., 53.

[22] Thudichum, loc. cit., 139.

cess of methyl alcohol added, with stirring, which precipitated the lead compound as a light yellow amorphous powder. This can be easily dried and is not hygroscopic. The first samples prepared seemed to have a nitrogen to phosphorus ratio of 1:2, but later preparations, carefully purified, had approximately the same ratio as the original cephalin. The material is purified by thoroughly extracting with boiling methyl alcohol, 80 per cent acetone, and dry acetone. Before purification a part of the lead salt is soluble in ether. The soluble and insoluble parts appeared to have the same composition. After precipitation of the ether-soluble fraction by adding about two volumes of methyl alcohol and extraction of the precipitate with dry acetone, the product is no longer ether-soluble. Thus the apparent solubility in ether was probably due to some impurity. The lead compound is insoluble in cold or warm alcohol, acetone, or ether, more or less soluble in benzene, toluene, and pyridine. Upon cooling the hot amyl alcohol solution deposits the unchanged lead salt as an amorphous mass. The amyl alcohol may be removed by washing with dry acetone. Glacial acetic acid does not completely dissolve the lead salt; it seems likely that a part of the lead is split off during the process of solution, since the residue from the extraction with acetic acid was found to contain lead acetate. A few of the many analyses are given below:

A. 0.1482 gm. substance gave 0.2154 gm. CO_2, 0.0790 gm. H_2O, and
0.0622 gm. ash.
0.5000 gm. substance neutralized 3.16 cc. $\frac{N}{10}$ HCl.
0.3000 "   "   gave 0.0252 gm. $Mg_2P_2O_7$.
B. 0.1498 "   "   " 0.2188 gm. CO_2, 0.0840 gm. H_2O, and 0.0634
gm. ash.
0.5000 gm.   "   neutralized 3.11 cc. $\frac{N}{10}$ HCl.
0.3000 "   "   gave 0.0294 gm. $Mg_2P_2O_7$.
C. 0.5000 "   "   neutralized 3.21 cc. $\frac{N}{10}$ HCl.
0.3000 "   "   gave 0.0262 gm. $Mg_2P_2O_7$.
D. 0.5000 "   "   neutralized 3.01 cc. $\frac{N}{10}$ HCl.
0.3000 "   "   gave 0.0276 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{74}NPO_4Pb_2$:	Calculated for $C_{42}H_{74}NPO_{12}Pb_2$:	A.	B.	C.	D.	Thudichum.
C.....	42.65	39.82	39.64	39.84			38.37
H.....	6.46	6.03	5.81	6.27			5.76
N.....	1.21	1.13	0.88	0.87	0.90	0.84	0.97
P.....	2.68	2.50	2.34	2.73	2.43	2.59	2.72
Ash...			41.97	42.40			

Preparation of Cephalin from the Lead Compound.

In order to determine whether the preparation and purification of the lead compound had brought about any purification of the cephalin itself, the lead compound was reconverted into the free cephalin. 50 gm. of the material were dissolved in a mixture of 200 cc. of toluene, 100 cc. of benzene, and 50 cc. of pyridine by warming a short time on the water bath. The lead was removed by means of hydrogen sulphide, the lead sulphide filtered off, and the clear solution concentrated to dryness in vacuum. The residue was taken up in ether and precipitated repeatedly with dry acetone. As the analyses show, this material had practically the same composition as the original cephalin used for making the lead salt; this indicates that the lead process is not one of purification.

0.1488 gm. substance gave 0.3276 gm. CO_2 and 0.1278 gm. H_2O.
0.5000 " " neutralized 6.84 cc. $\frac{N}{10}$ HCl.
0.3000 " " gave 0.0388 gm. $Mg_2P_2O_7$.

	C	H	N	P
Found:	60.05	9.61	1.87	3.60

This process is, at best, very unsatisfactory. The preparation of the lead salt is not quantitative, and the removal of the lead is accompanied by great loss of material. The operations must be carried out as rapidly as possible, for if cephalin or its lead compound is allowed to stand for some time in a benzene-pyridine solution, even at 0°, a certain decomposition occurs, in which the nitrogen content is increased. The nature of this change is being further investigated.

Two samples of cephalin, purified through the lead salt, were again converted into the lead compound as given above. The same lead compound was obtained, again showing that the cephalin thus obtained was identical with that used for the preparation of the original lead salts.

0.5000 gm. substance neutralized 3.4 cc. $\frac{N}{10}$ HCl.
0.5000 " " " 3.46 " " "
0.3000 " " gave 0.0242 gm. $Mg_2P_2O_7$.
0.3000 " " " 0.0260 " "

	N		P	
Found:	0.95	0.97	2.24	2.41

Quantitative Hydrolysis.

As the base and the glycerophosphoric acid of cephalin are water-soluble, it is comparatively easy to determine the amount of fatty acid obtained upon hydrolysis. 5 gm. of cephalin were boiled with 300 cc. of 3 per cent sulphuric acid for 24 hours. Upon cooling 0°, the fatty acids solidified. These were filtered off, washed free of acid by melting over water several times, and then taken up in dry acetone. By evaporating off the acetone several times the product was dehydrated. It was finally dried to constant weight at 100°.

4.75 gm. cephalin gave 2.970 gm. fatty acid.
2.75 " " " 3.026 " " "
5.0 " " " 3.202 " " "

	Fatty acid. *per cent*	
Found:	62.6 63.7	64.0

Glycerol Estimations.

Koch believed that cephalin contained one methyl group attached to nitrogen, because when heated with hydriodic acid one molecule of methyl iodide was formed under 240°. Similar results were obtained by Fränkel and Neubauer. Renall also reports the formation of methyl iodide when cephalin was heated to 360°, but states that the amount obtained was not equivalent to the ratio N:Me = 1:1. Recent work makes it appear evident that this formation of methyl iodide was really due to the presence of glycerol.

Foster[23] has shown that glycerol may be readily determined in lipoids by the use of the original Fanto-Zeisel method. By using a modified Herzig-Meyer apparatus it is possible to determine both the glycerol and the methyl attached to nitrogen.

We have used the Fanto-Zeisel method for the determination of glycerol in cephalin with satisfactory results. The reaction proceeds smoothly, with little or no foaming, and is complete in about 2 hours if the temperature of the metal bath is maintained at 125–128°. Blank determinations on a known solution of glycerol in water gave good checks. Experiments with aminoethyl

[23] Foster, M. L., *Jour. Biol. Chem.*, 1915, xx, 403.

alcohol showed that under the conditions of the reaction no decomposition (with the formation of volatile iodides) took place. 0.3000 gm. of cephalin gave, in three experiments, 0.0748, 0.0760, and 0.0784 gm. of silver iodide respectively. This corresponds to 9.77, 9.93, and 10.2 per cent glycerol. Koch's result, as recalculated by Foster, was 10.8 per cent, while Winterstein and Hiestand found 10.2 per cent. Foster obtained a slightly lower figure, 8.21 per cent. The corresponding figure for lecithin varies from 8.75 to 11.6 per cent glycerol.

Composition of the Cephalin Molecule.

The amount of base (aminoethyl alcohol) in cephalin cannot be estimated directly, because all its derivatives (gold chloride salt, platinic chloride salt, picrate, picrolonate) are very soluble in water or alcohol. However, since all the nitrogen has been shown to be amino nitrogen,[24] we are justified in using the percentage of nitrogen as a basis for the calculation of the amount of base. With 1.9 as the average of the nitrogen content, the amount of aminoethyl alcohol is calculated as 8.26 per cent.

In the same way, with 3.85 as the average phosphorus content, the amount of phosphoric acid is calculated as 12.17 per cent.

In calculating the composition of the molecule the components should add up to 109.7 per cent, since this is the relation between the molecular weight of the formula $C_{41}H_{78}NPO_8$ and the hydrated formula $C_{41}H_{86}NPO_{12}$. The calculated and found values are given below:

	Calculated for $C_{41}H_{78}NPO_8$:	Found:
Base	8.2	8.26
Glycerol	12.4	10.00
Phosphoric acid	13.2	12.17
Fatty acid	75.9	63.40
Totals	109.7	93.83

Thus it is seen, as mentioned in the introduction, that the molecule is not entirely accounted for. As yet we can give no reason for the discrepancy.

[24] Baumann, *Biochem. Z.*, 1913, liv, 30. Renall, *ibid.*, 1913, lv, 296.

Hydrocephalin.

1 gm. of cephalin was dissolved in a mixture of 40 cc. of ordinary ether and 5 cc. of glacial acetic acid and shaken with colloidal palladium in an atmosphere of hydrogen, according to Paal. The hydrogen was absorbed at the rate of about 1 cc. per minute, gradually decreasing as the reaction proceeded. At the end of the reaction 70 cc. (measured at room temperature) had been absorbed, while 1 gm. of cephalin, with the formula $C_{41}H_{78}NPO_{13}$ (mol. wt. 743) should absorb 60 cc. measured under standard conditions. Reduction experiments in pyridine and acetic acid or in amyl alcohol and acetic acid were unsuccessful. The reaction product was concentrated on the water bath until all the ether had been removed, then treated with a large volume of acetone and filtered. The precipitate was taken up in boiling absolute alcohol containing a trace of acetic acid, heated until the palladium was coagulated, and filtered. Upon cooling the reduced cephalin separated out as an amorphous powder. This was purified by washing with ether, dissolving in boiling absolute alcohol, and allowing the product to settle out at 0°. It forms a nearly colorless, non-hygroscopic powder. Because of the small amount at our command we were unable to obtain it crystalline.

Two samples were analyzed. The first was analyzed after the second and third crystallizations, the second after the second crystallization.

A. 0.0998 gm. substance gave 0.2273 gm. CO_2 and 0.0930 gm. H_2O.
   0.1062 "   "   "   0.2420 "   "   "   0.0958 "   "
   0.0300 "   "   "   0.0700 "   "   "   AgI (glycerol estimation).
B. 0.1060 "   "   "   0.2412 "   "   "   0.1004 gm. H_2O.

	C	H	Glycerol.
Found: A.	62.12	10.43	9.15
	62.15	10.10	
B.	62.06	10.60	

The two samples given above were prepared last year. This fall we again took up the question of obtaining hydrocephalin, but with no better success. The reaction appears to be uncertain; at times the reduction proceeds smoothly, at other times

scarcely any hydrogen is absorbed. We have tried to further purify the cephalin but this does not increase the rate of reduction.

The product obtained this year gave the following figures on analysis:

0.1022 gm. substance gave 0.2256 gm. CO_2 and 0.0912 gm. H_2O.
0.300 "   "   "   0.0370 " $Mg_2P_2O_7$.
0.200 "   "   neutralized 3.01 cc. $\frac{N}{10}$ HCl.

	Found:
C	62.25
H	10.34
N	2.22
P	3.62

GLUCOSAMINOHEPTONIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The work of Levene and LaForge[1] has demonstrated that the substitution of the amino group by a hydroxyl in the series of amino sugars may be associated with a change of configuration of the α-carbon atom. This property raises great difficulty in reaching, by simple methods, a conclusion regarding the complete configuration of any amino sugar. The configuration of glucosamine, the most accessible and the best known substance of this group, still remains unknown. Because of the difficulty of arriving at the goal by direct methods, the problem is being approached in this laboratory by several indirect ways, all of which will be discussed later. One of these, however, will be outlined here. In the light of the experience of Fischer[2] and his coworkers it seems possible that a substitution in the β position from the carbonyl group may take place without a Walden rearrangement. If this were to hold true for the 2-aminoheptonic acids, then the solution of the question of the configuration of these substances should be easily accessible. On the other hand, the knowledge of the configuration of the carbon atom carrying the amino group in the heptonic acids will contain in itself the information regarding the configuration of the same group in the parent hexosamines. Hence the first problem demanding solution is the question of the occurrence or non-occurrence of the Walden rearrangement during deamination of the 2-aminoheptonic acids. The derivative of glucosamine was the subject of the present investigation. In order to make this investigation

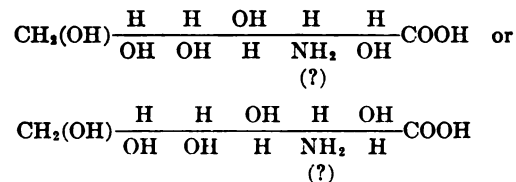[1] Levene, P. A., and LaForge, F. B., *Jour. Biol. Chem.*, 1915, xx, 433; xxi, 345, 351; xxii, 331.

[2] Fischer, E., and Scheibler, H., *Ber. d. chem. Ges.*, 1909, xlii, 1219. Fischer, E., Scheibler, H., and Groh, R., *ibid.*, 1910, xliii, 2020.

possible it is necessary to be in possession of a crystalline 2-amino-heptonic acid derived from glucosamine.

An attempt to prepare this heptonic acid was made by Neuberg,[2] who claimed to have obtained an amorphous copper salt, which gave analytical values agreeing with those required by the heptonic acid. Such material could be of little value in our work, especially since the amorphous salts prepared by us gave values only approximating those required by the theory. Hence we renewed the efforts towards obtaining a crystalline glucosamino-heptonic acid. The present communication contains a report of the mode of preparation and of the properties of the acid which has the structure of one of the following two possible epimers.

$$CH_2(OH)\dfrac{H \quad H \quad OH \quad H \quad H}{OH \quad OH \quad H \quad NH_2 \quad OH}COOH \quad \text{or}$$
$$(?)$$

$$CH_2(OH)\dfrac{H \quad H \quad OH \quad H \quad OH}{OH \quad OH \quad H \quad NH_2 \quad H}COOH$$
$$(?)$$

EXPERIMENTAL PART.

Portions of 25.0 gm. of glucosamine hydrochloride were taken up in 25 cc. of water, 10 cc. of an 80 per cent aqueous solution of hydrocyanic acid added, and this was followed by the addition of 12.5 cc. of ammonia water. The chloride generally dissolved soon after the addition of ammonia. The solution turned dark brown within 2 hours, and on further standing became quite viscous. It was allowed to stand at room temperature for 3 to 4 days. The solution was then taken up in about 400 cc. of water, boiled with a slight excess of lead carbonate, filtered, and precipitated with basic lead acetate and ammonia. The precipitate was decomposed with hydrogen sulphide, filtered, and concentrated to about 300 cc. To the solution about 40.0 gm. of barium hydroxide were added and the mixture was heated over a flame until the greatest part of ammonia was removed. On standing, a brown flocculent precipitate formed and was removed by filtration. The filtrate was then concentrated under

[2] Neuberg, C., *Ber. d. chem. Ges.*, 1902, xxxv, 4009. Neuberg, C., and Wolff, H., *ibid.*, 1903, xxxvi, 618.

diminished pressure to dryness to remove all the ammonia. The residue was taken up in water, and the solution acidulated with sulphuric acid to remove all barium. Omitting filtration, excess of lead carbonate was added and the mixture boiled until, upon a further addition of the reagent, evolution of carbon dioxide was no longer manifested. The reaction product was then allowed to stand at 0°C. over night and filtered. The lead was then removed by means of hydrogen sulphide, the remaining hydrochloric acid by means of silver carbonate, and the excess of silver again removed by hydrogen sulphide. The filtrate was boiled with animal charcoal until it became perfectly colorless. The colorless solution was concentrated to about 75.0 cc. and to this methyl alcohol was added in small portions. The addition of alcohol caused the appearance of a white flocculent precipitate at the place of contact. By manipulation this was slowly brought again in solution, and the addition of alcohol continued until the solution turned slightly opalescent. It was then allowed to stand at least 24 hours at 0°C. A crystalline precipitate appeared, consisting of rosettes composed of needles. The yield was about 30 per cent of the glucosamine hydrochloride used. On recrystallization the substance crystallized in individual needles. The acid has a melting point of 169°C. (corrected). For analysis it was dried in a vacuum desiccator over sulphuric acid at 100°.

0.1004 gm. of substance gave 0.1366 gm. CO_2 and 0.0618 gm. H_2O.
0.0970 " " " required for neutralization 4.38 cc. $\frac{N}{10}$ acid (Kjeldahl).

	Calculated for $C_7H_{13}NO_7$:	Found:
C	37.33	37.10
H	6.66	6.88
N	6.22	6.25

In 2.5 per cent solution of hydrochloric acid the substance had the following rotation:

Initial.

Equilibrium. (After 24 hours.)

$$[\alpha]_D^{25} = \frac{-0.02° \times 2.168}{0.1 \times 0.150} = -2.89° \qquad [\alpha]_D^{25} = \frac{-0.52° \times 2.168}{0.1 \times 0.150} = -7.52°$$
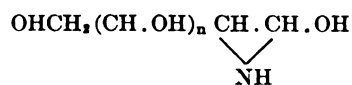
AMMONIA DERIVATIVES OF THE SUGARS.

By P. A. LEVENE.

The structure of the so called glucosimines has been the subject of considerable discussion. Lobry de Bruyn,[1] who first discovered them, assigned to them the following structure:

$$OHCH_2(CH.OH)_n \underset{\diagdown \diagup}{CH.CH.OH}$$
$$NH$$

Wohl[2] modified this first conception, accepting the ring between the end and the γ-carbon atoms:

$$OHCH_2CH(OH)CH.CH(OH)CH(OH)CH.OH$$
$$\underline{\qquad\qquad NH \qquad\qquad}$$
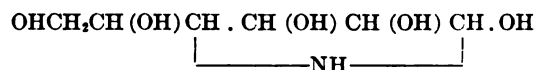
Finally, Irvine, Thomson, and Garret[3] have assumed the glucosidic structure:

$$OH\,CH_2CH(OH)CH.CH(OH)CH(OH)CH.NH_2$$
$$\underline{\qquad\qquad O \qquad\qquad}$$
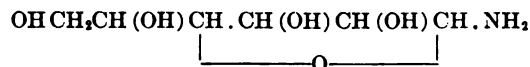
The evidence advanced by Irvine and his coworkers is of indirect nature, and more direct evidence supporting their hypothesis seemed desirable. Besides, the knowledge of the structure of these substances has assumed new importance because

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[1] Lobry de Bruyn, C. A., and Franchimont, A. P. N., *Rec. d. trav. chim. d. Pays-bas*, 1893, xii, 286. Lobry de Bruyn. *Ber. d. chem. Ges.*, 1895, xxviii, 3082.

[2] Wohl, quoted by von Lippmann, E. O., Die Chemie der Zuckerarten, Braunschweig, 1904, i, 504.

[3] Irvine, J. C., Thomson, R. F., and Garret, C. S., *Jour. Chem. Soc.*, 1913, ciii, 238.

of its bearing on the mechanism of the reaction between hydro-cyanic acid and the sugar imines.

In order to prove the glucosidic structure it is necessary to furnish conclusive evidence as to one of two requisites, or to both: first, as to the existence of the γ-oxidic ring in sugars (this structure carries the property of mutarotation); second, as to the presence of an unsubstituted NH_2 group. These two points have been taken into consideration by previous workers, but they received no conclusive answer.

The presence of a primary NH_2 group was tested in this work by comparing the nitrogen values obtained by the Kjeldahl process and by the method of Van Slyke with nitrous acid. It was found that in 4 minutes all the nitrogen of the imine was transformed into nitrogen gas. In the same interval ammonia does not give off more than 20 per cent of its nitrogen as nitrogen gas.[4]

Furthermore, on material which was only slightly hydrolyzed, the values obtained by the Kjeldahl process were higher than those obtained in 4 minutes by the nitrous acid method. In connection with this it may be mentioned that the rate of hydrolysis varies with individual sugars. The derivatives of four sugars were analyzed: glucose, galactose, xylose, and lyxose. Lyxosimine was found the most, and glucosimine the least stable.

Regarding the change in rotation the following was noted. Freshly prepared samples of xylosimine and of lyxosimine, kept in aqueous solution at 0° for 24 hours, did not manifest any change in rotation. In 63 hours there was observed a slight change in rotation and with it there was also noted a change in nitrogen distribution, the nitrogen gas evolved by nitrous acid showing a lower value than the total nitrogen.

On the other hand, if the sample of the dry imine was kept for 7 days over soda lime under diminished pressure and then analyzed, it was found to manifest some mutarotation in 24 hours, even when the nitrogen distribution remained constant. In this sample there was always present a slight excess of the total nitrogen over the amino nitrogen. Hence the mutarotation was undoubtedly due to the presence of little of the free sugar.

[4] Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 281.

Thus, the presence of the primary amino group in the molecule of the sugar imines is demonstrated; on the basis of this one is justified in assigning to the sugar imines a glucosidic structure. The name amino-glucosides would perhaps be more correct.

Table of Results.

	Total N calculated for $C_5H_{11}NO_4$ = 9.40 per cent. Found:		NH$_2$ N calculated for $C_5H_9O_4.NH_2$ = 9.40 per cent. Found:		
	Immediately.	After 63 hrs.	Immediately.	After 24 hrs.	After 63 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Xylosimine.					
I...............	9.73	9.64	9.39		9.07
II...............	9.06		8.78	8.78	
Lyxosimine.					
I...............	9.64	9.58	9.16		8.38
II...............	8.92		7.83	7.80	

EXPERIMENTAL PART.

I. Xylosimine[5] was prepared according to the method of Lobry de Bruyn and Van Leent.[6] The substance was dried for 24 hours over soda lime under diminished pressure.

0.1000 gm. substance required for neutralization 6.95 cc. $\frac{N}{10}$ acid (Kjeldahl).

0.0500 gm. substance gave in 4 minutes 8.2 cc. N at 20°,764 mm. (Van Slyke).

The same solution was analyzed after 63 hours' standing at 0°C.

0.1000 gm. substance required for neutralization 6.89 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 7.9 cc. N gas at 20°, 765 mm.

The rotation of the substance in cold water of about 5°C. (dissolved in water at 0°) was the following:

Immediately.

$$[\alpha]_D^3 = \frac{-0.38° \times 4.1850}{0.5 \times 0.2000} = -15.9°$$

After 63 hours.

$$[\alpha]_D^3 = \frac{-0.38° \times 4.1850}{0.5 \times 0.2000} = -15.9°$$

---

[5] We take this occasion to express our indebtedness to the Lookout Refining Company, Chattanooga, Tenn., who furnished us free of charge the cottonseed hulls which served for the preparation of the xylose used in this work as well as in the work on "Xylohexosaminic Acid."

[6] Lobry de Bruyn, C. A., and Van Leent, F. H., *Rec. d. trav. chim. d. Pays-bas*, 1895, xiv, 134.

II. Xylosimine was prepared in the same manner and kept for 7 days in a desiccator.

0.1000 gm. substance required for neutralization 6.47 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 7.7 cc. N gas at 20°, 762 mm.

After 24 hours the same solution gave:

0.0500 gm. substance gave in 4 minutes 7.6 cc. N gas at 19°, 768 mm.

Rotation under the same conditions as above:

<table>
<tr><td align="center">Immediately.</td><td align="center">After 24 hours.</td></tr>
</table>

$$[\alpha]_D^5 = \frac{-0.42° \times 4.1630}{0.5 \times 0.2000} = -17.48° \qquad [\alpha]_D^5 = \frac{-0.38° \times 4.1630}{0.5 \times 0.2000} = -15.71°$$

III. Lyxosimine was prepared in the manner described by Levene and La Forge.[7] It was kept in the desiccator over night.

0.1000 gm. substance required for neutralization 6.89 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 8.0 cc. N gas at 20°, 764 mm.

The same solution after 63 hours gave:

0.1000 gm. substance required for neutralization 6.84 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 7.3 cc. N gas at 20°, 765 mm.

The rotation was:

<table>
<tr><td align="center">Immediately.</td><td align="center">After 63 hours.</td></tr>
</table>

$$[\alpha]_D^5 = \frac{-1.30° \times 4.1950}{0.5 \times 0.2000} = -54.53° \qquad [\alpha]_D^5 = \frac{-1.06° \times 4.1950}{0.5 \times 0.2000} = -44.46°$$

IV. Lyxosimine was prepared as the sample above, but kept in the desiccator 7 days.

0.1000 gm. substance required for neutralization 6.37 cc. $\frac{N}{10}$ acid.

0.0500 "   "   gave in 4 minutes 6.75 cc. N gas at 19°, 768 mm.

The rotation was:

<table>
<tr><td align="center">Immediately.</td><td align="center">After 24 hours.</td></tr>
</table>

$$[\alpha]_D^5 = \frac{-0.72° \times 4.1952}{0.5 \times 0.2000} = -30.25° \qquad [\alpha]_D^5 = \frac{-0.55° \times 4.1952}{0.5 \times 0.2000} = -23.10°$$

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[7] Levene, P. A., and La Forge, F. B., *Jour. Biol. Chem.*, 1915, xxii, 331.

SPHINGOSINE.

IV. SOME DERIVATIVES OF SPHINGOSINE AND DIHYDROSPHINGOSINE.

By P. A. LEVENE and C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Sphingosine has generally been identified by the analysis of the sulphate. Some workers have based their conclusions on the presence of sphingosine when the sample possessed the nitrogen content of sphingosine sulphate or, besides the nitrogen content, showed the required melting point. In the course of the work carried out in this laboratory during the last few years the conviction was gained that the composition of the sulphate is not sufficiently constant to justify conclusions on the basis of analytical data for one or two elements. Thus the differences in the nitrogen and sulphur content of the homologues of sphingosine are within the limits of analytical error. Also the melting point does not offer a sufficient guarantee of its purity. Because of this, attempts were made to prepare other salts of sphingosine and dihydrosphingosine. Picric and picrolonic acids were found to give with the two bases salts of constant composition. However, the solubility of these salts in organic solvents is too great to make them very useful for analytical purposes.

This communication contains also a report of further work on the structure of sphingosine. Levene and West[1] have shown that the probable structure of sphingosine is

$$C_{12}H_{25}CH = CH . CHOH . CHOH . CH_2NH_2$$

The isolation of normal pentadecylic acid from the oxidation products of dihydrosphingosine established the nature of the first 15 carbon atoms. There thus remains as unknown the character

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913-14, xvi, 549; 1914, xviii, 481.

of the two end carbon atoms and the relative positions of the hydroxyl and amino groups.   Levene and Jacobs[2] first attempted to answer these questions by trying to reduce dihydrosphingosine to the corresponding amine.   Using hydriodic acid in sealed tubes at 125°, they obtained an unsaturated compound, sphingamine, $C_{17}H_{35}N$.   The same substance was also obtained when the dichloro derivative was reduced with sodium and alcohol. Because of this it was attempted to modify the conditions of reduction of the base by means of hydriodic acid.   An interesting observation was made when the process was carried out in glacial acetic acid solution; under these conditions only one hydroxyl group was reduced, leaving a monohydroxymonoamino alcohol.   This substance undoubtedly could serve as a starting point for further work on the structure of sphingosine.   However, a chance discovery made the material more accessible in another way.

In the course of the present work also the trihydroxy alcohol— dihydrosphingosol—was obtained.

EXPERIMENTAL PART.

Sphingosine Picrolonate.

The sphingosine from 4.5 gm. of sphingosine sulphate, dissolved in a little alcohol, was added to a solution of 3 gm. of picrolonic acid in alcohol.   The slightly yellow solution immediately turned a deep red.   Boiling water was then added until the solution was turbid; upon cooling the picrolonate separated as a deep red oil, which gradually changed to yellow crystals.   It was purified by precipitating several times from an alcoholic solution with boiling water and finally by extracting with ether at room temperature.   Sphingosine picrolonate is easily soluble in ethyl alcohol, somewhat soluble in methyl alcohol and acetone, and very slightly soluble in ether.   It softens at 81° and melts at 87–89°.

0.1110 gm. substance gave 0.2397 gm. CO_2 and 0.0792 gm. H_2O.

	Calculated for $C_{17}H_{43}N_5O_7$:	Found:
C	58.97	58.90
H	7.88	7.98

[2] Levene, P. A., and Jacobs, W. A., *Jour. Biol. Chem.*, 1912, xi, 547.

Abderhalden and Weil[3] report that they were able to estimate picrolonates by the ordinary Kjeldahl method, only two of the nitrogens of picrolonic acid being converted into ammonia. We tried this method on the above picrolonate but found that the value for N was always high. The following results were obtained:

0.2434 gm. substance required 17.8 cc. $\frac{N}{10}$ HCl.
0.2816 "   "   "   20.4 "   "   "
0.2021 "   "   "   14.7 "   "   "

	Calculated for 3N:		Found:	
N	7.65	10.24	10.15	10.19

The determination of nitrogen in dl-leucine picrolonate[4] was then tried, for which Abderhalden and Weil give: calculated, 10.65; found, 10.90.

0.1924 gm. substance required 17.6 cc. $\frac{N}{10}$ HCl.
0.1777 "   "   "   15.7 "   "   "

	Calculated for 3N:	Found:	
N	10.65	12.81	12.38

Blank experiments on picrolonic acid gave values between those calculated for 2N and 3N.

Dibromosphingosine Sulphate.

Sphingosine sulphate, dissolved in chloroform, was treated with a dilute solution of bromine in chloroform at room temperature until the bromine color was permanent. The mixture was allowed to stand several hours, the chloroform removed on the steam bath, and the product washed with water and dilute sodium bisulphite solution. After drying it was recrystallized from acetic acid. Dibromosphingosine sulphate forms a light gray crystalline powder, insoluble in alcohol and ether, soluble in acetic acid and chloroform.

0.1200 gm. substance gave 0.1811 gm. CO_2 and 0.0793 gm. H_2O.

	Calculated for $(C_{17}H_{34}NO_2Br_2)_2H_2SO_4$:	Found:
C	41.30	41.10
H	7.34	7.40

[3] Abderhalden, E., and Weil, A., Ztschr. f. physiol. Chem., 1912, lxxviii, 150.
[4] Levene, P. A., and Van Slyke, D. D.. Jour. Biol. Chem., 1912, xii, 127.

Dihydrosphingosine Sulphate.

This was prepared by the method already described by Levene
and Jacobs.   It was found in many experiments, especially if the
ether contained alcohol, that the bulk of the dihydro base re-
mained in the acetic acid-water solution with the palladium.   It
was isolated as follows: The ether layer was removed from the
acetic acid and water.   The ether was concentrated and the resi-
due added to the dilute acid solution; this was then warmed and
filtered to remove the palladium, and the filtrate treated with 10
per cent sulphuric acid as long as the sulphate separated.   This
was thoroughly cooled, filtered, and recrystallized from dilute
alcohol (80 per cent).   Dihydrosphingosine sulphate differs from
that of sphingosine in that it is much less soluble in absolute
alcohol and in chloroform.

Dihydrosphingosine Picrolonate.

This was prepared in the same way as the sphingosine picrolo-
nate.   It crystallizes in long, slender, yellow needles, which begin
to soften at 110° and melt at 120–121°.

0.1317 gm. substance gave 0.2832 gm. CO_2 and 0.0966 gm. H_2O.

	Calculated for $C_{27}H_{45}N_4O_7$:	Found:
C	58.76	58.79
H	8.22	8.23

Dihydrosphingosine Picrate.

2 gm. of the dihydro base in 25 cc. of hot alcohol were treated
with 25 cc. of a saturated alcoholic solution of picric acid, and hot
water was added until the solution was turbid.   The yellow pre-
cipitate was purified by recrystallization from dilute alcohol and
finally by extraction with ether.   The picrate forms pure yellow
crystals, which melt at 88–89°.

0.1096 gm. substance gave 0.2150 gm. CO_2 and 0.0762 gm. H_2O.

	Calculated for $C_{24}H_{40}N_4O_9$:	Found:
C	53.44	53.50
H	7.80	7.78

Dihydrosphingosol (*Trihydroxyheptyldecane*) $C_{17}H_{33}(OH)_3$.

Dihydrosphingosine sulphate was dissolved in boiling acetic acid, cooled to room temperature, and gradually treated with slightly more than the theoretical amount of solid sodium nitrite. A concentrated aqueous solution of nitrite may also be used. After standing several hours the reaction product was concentrated to a small volume, poured into a large quantity of ice water, the precipitate filtered off and recrystallized several times from a small quantity of acetone, finally with the use of animal charcoal. *Dihydrosphingosol* is a colorless, crystalline solid, melting at 54-55° and easily soluble in all organic solvents.

	Calculated for $C_{17}H_{36}O_3$:	Found:
C.	70.01	70.76
H.	12.49	12.58

Hydroxyheptadecylamine, $C_{17}H_{34}(OH)NH_2$.

Hydroxyheptadecylamine was obtained as the reaction product of hydriodic acid upon an acetic acid solution of dihydrosphingosine sulphate either at 100° or at the boiling point of the mixture. The only influence the time of heating had was to decrease the yield, the maximum yield (which was always small) being obtained by 5 to 7 hours' heating. One experiment in which the heating was carried out for 20 hours gave the hydroxy-amine as the only basic reaction product. The other product of this reaction appears to be a hydrocarbon or alcohol, produced by the hydrolysis of the amino group.

10 gm. of dihydrosphingosine sulphate were dissolved in a mixture of 50 cc. of hydriodic acid and 200 cc. of glacial acetic acid, about 1 gm. of phosphonium iodide was added, and the solution heated in a boiling water bath for 5 to 7 hours. The solution was then concentrated in vacuum to a thick syrup, the residue washed with water and taken up in absolute alcohol. Alcoholic hydrochloric acid was added to the solution and the mixture gradually treated with metallic zinc until colorless. This colorless solution was concentrated in vacuum to a small volume and the salts, a mixture of the chloride and iodide, were precipitated with cold water. The free base was obtained by neutralizing the alcoholic

solution of the salts with sodium methylate, evaporating to dryness, and extracting with ether. This was changed into the sulphate by warming with dilute sulphuric acid, the crude product recrystallized from acetone, the non-basic part removed by washing with ether, and the sulphate finally recrystallized from absolute alcohol. It separates as long, slender, colorless needles, forming into rosettes, which melt at 206–208°. Many samples were analyzed, of which two are reported here:

0.1082 gm. substance gave 0.2528 gm. CO_2 and 0.1158 gm. H_2O.
0.1226 "   "   "   0.2893 "   "   " 0.1306 "   "

	Calculated for $(C_{17}H_{37}O)_2H_2SO_4$:	Found:	
C	63.69	63.43	63.45
H	11.96	11.98	11.93

1.5 gm. of the sulphate were transformed into the free base. For this purpose it was dissolved in methyl alcohol. To the alcoholic solution was added a solution of barium hydroxide in methyl alcohol until it reacted alkaline to phenolphthalein. Acetone was then added to remove excess of barium, the filtrate concentrated in vacuum and recrystallized out of alcohol. The free base crystallized out in beautiful scales of the appearance of hydroxyheptadecylamine.[5] It melts at 85.5°.

The analysis of the substance was as follows:

0.1018 gm. substance gave 0.2756 gm. CO_2 and 0.1220 gm. H_2O.

	Calculated for $C_{17}H_{34}(OH)NH_2$:	Found:
C	75.3	74.12
H	13.68	13.47

The analysis shows the presence of a minimal quantity of dihydrosphingosine in the sample of the reduced base. This undoubtedly could be removed by repeated recrystallization.

[5] Levene, P. A., *Jour. Biol. Chem.*, 1915, xxiv, 79.

SPHINGOMYELIN. III.

By P. A. LEVENE.[1]

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Sphingomyelin was discovered by Thudichum.[2] This investigator established the principal distinction of this phosphatide from those of the lecithin group; namely, the absence of glycerol in its molecule. All other statements of the discoverer regarding sphingomyelin needed revision, since they were based on analysis of material that was not a pure phosphatide or pure phosphatides, but a mixture of these with cerebrosides. Rosenheim and Tebb[3] later improved the method of preparation of sphingomyelin, and had in their possession a much purer material. In a general way they accepted Thudichum's conclusions regarding the structure of the substance. Other investigators who, in course of their work on phosphatides, encountered the substance always dealt with grossly contaminated material.

In a previous communication[1] the writer reported a mode of procedure for preparing sphingomyelin free from cerebrosides, and also the conditions for the hydrolysis of the substance. The following substances were then obtained: (1) phosphoric acid; (2) two fatty acids, cerebronic and lignoceric; (3) three basic substances, choline, sphingosine, and a base of the composition $C_{17}H_{35}NO$. Sphingosine had not been isolated in pure form, and the composition of the third base was rather suggested than definitely demonstrated. Since that publication the work has been continued and extended to the sphingomyelin obtained from other organs; namely, kidney, liver, and the yolk of hen's

[1] Levene, P. A., *Jour. Biol. Chem.*, 1913, xv, 153; 1914, xviii, 453.

[2] Thudichum, J. L. W., The Chemical Constitution of the Brain, London, 1884, 105.

[3] Rosenheim, O., and Tebb, M. C., *Quart. Jour. Physiol.*, 1908, i, 297; *Jour. Physiol.*, 1910, xli, p. i.

egg. The method of preparation of the substance was slightly
modified with the result that the sphingomyelin employed in the
present work was entirely free from contaminating cerebrin.

The elementary composition and the specific optical rotation of
the new material were quite constant.   Sphingomyelin is micro-
crystalline in nature.   /

Employing the old method of hydrolysis the products obtained
from the new material differed from those recorded in the earlier
work only in the point of the acids.   Cerebronic and lignoceric
were the two previously recorded.   Cerebronic acid could not
be detected in the course of the present work.   In the older mate-
rial it undoubtedly originated in the adhering cerebrin.   Lignoceric
acid was identified beyond doubt, and with it was demon-
strated the presence of another acid, of lower molecular weight.
From the present data it seems to be a hydroxy acid.   The mixed
acids obtained on hydrolysis of sphingomyelin contain at least
50 per cent of the new acid.   However, it was not prepared in
a state of satisfactory purity through any of the conventional
methods of fractionation, either through fractional crystalli-
zation from organic solvents, through the sodium, lithium, or
magnesium salts, or through fractional distillation of the esters.
It is hoped, however, that with a larger quantity of material on
hand the difficulty will be overcome.

For the bases the old method of hydrolysis gave results which in
every way substantiated the older conclusions.   Again choline,
sphingosine, and a base $C_{17}H_{35}NO$ were identified.   The assumed
composition of $C_{17}H_{35}NO$ was demonstrated to be the correct one;
and after reduction with hydrogen and palladium it was shown to
possess the structure of hydroxyheptadecylamine $C_{17}H_{34}(OH).NH_2$.
The latter base gave up, in 10 minutes, all its nitrogen in the form
of nitrogen gas on treatment with nitrous acid in the Van Slyke
apparatus, and, further, it formed a diacetyl derivative.   The
reduced base will be referred to as sphingine.   In the free state
it crystallizes in beautiful plates that have the appearance of
cholesterol.   However, in the course of the work sufficient evidence
accumulated for the belief that the base was a secondary product
formed from sphingosine.   In the process of hydrolysis of sphing-
omyelin with alcohol and water sphingosine loses a molecule of
water, giving rise to a base of the nature of anhydrosphingosine,

$$C_{12}H_{23}CH = CHCH = C(OH)CH_2NH_2.$$
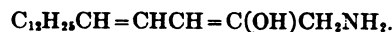
. The position of the additional double bond in this graphic formula is arbitrary. Owing to the great solubility of the free base or of its sulphate in organic solvents it generally escaped detection, but it always contaminated the sphingosine prepared from sphingomyelin. It was perhaps due to this impurity that Thudichum never succeeded in isolating pure sphingosine from sphingomyelin. He referred to the base isolated by him as an impure sphingosine or a homologue of sphingosine.

In our own work the base escaped detection for a long time. Only after the mixed bases were reduced by hydrogen in the presence of colloidal palladium could their separation be accomplished; namely, through the fractional crystallization of their sulphates from a solution of equal parts of chloroform and amyl alcohol.

The reasons for the belief in the secondary origin of anhydrosphingosine are the following: when the cleavage of sphingomyelin was accomplished through hydrolysis in sealed tubes with 3 per cent sulphuric acid solution, anhydrosphingosine could not be detected, and sphingosine was easily isolated in pure condition. On the other hand, when a sample of pure cerebrin—corresponding to Thierfelder's cerebron—was hydrolyzed by the combined method and the bases were subsequently reduced, in place of dihydrosphingosine, pure sphingine was obtained. There should be recorded here another difficulty in identifying sphingosine. The sulphate which generally serves for identification of the base on recrystallization easily changes its composition, a part of the neutral salts changing into either the basic or the acid salt. The same applies to sphingine. It was therefore found necessary to purify sphingosine and more so dihydrosphingosine and sphingine by converting them into the free bases; these may then be reconverted into the sulphate if desired.
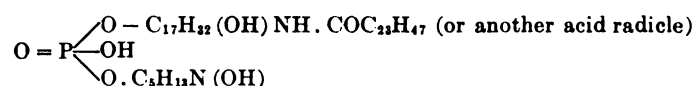
Thus, for the present, sphingomyelin was found to yield on hydrolysis phosphoric acid, two fatty acids, and two bases, choline and sphingosine.

Thudichum and Rosenheim and Tebb accepted in the molecule of sphingomyelin besides the bases and the acids also an alcohol. This view does not agree with the analytical finding of the present work; namely, the cleavage of our material yielded 34 per cent of

its weight in the form of sphingosine and 43 per cent in the form of the organic acids. These values leave no room in the molecule of the phosphatide for the alcohol sphingol, and, on the other hand, they agree with the assumption that a monophosphatide of the sphingomyelin type is composed of single equivalent proportions of the components isolated by us.

Hence, for the present one is justified in assuming for a phosphatide of this type the structural expression,

$$O = P\begin{cases} O - C_{17}H_{32}(OH)\,NH.\,COC_{23}H_{47}\ \text{(or another acid radicle)} \\ OH \\ O.\,C_5H_{13}N\,(OH) \end{cases}$$

This assumption is further justified by a chance discovery of an intermediary product. This was obtained on hydrolysis of a sample of kidney sphingomyelin. It possessed the following properties: it contained nitrogen, gave no nitrogen gas on treatment with nitrous acid, did not form salts with mineral acids, and had the elementary composition of lignoceryl sphingine, $C_{17}H_{34}(OH)NH.CO.C_{23}H_{47}$. Lack of material prevented hydrolysis of the substance.

Whether sphingomyelin is a mixture of two phosphatides of the described type, or a diphosphatide, can for the present not be stated with certainty. This question as well as the question of the structure of the second fatty acid is under investigation.

Regarding the relation of the sphingomyelins derived from different organs, one is inclined to believe in their identity. If ultimately it will be demonstrated that sphingomyelin is a mixture of two monophosphatides, then it may appear that their proportion varies somewhat in the material obtained from different organs. The following table of the elementary analysis and of the physical properties of samples derived from different sources brings out best the justification for the expressed belief.

Source.	C	H	N	P	$[\alpha]_D$	Sphingosine.	Acids.
						per cent	per cent
Brain.............	66.59	11.26	3.78	3.99	+8.20 +7.53	34.10	43.00
Kidney.............	64.80	11.41	3.50	3.82	+8.73	32.10	49.00
Liver...............	64.47	11.57	3.41	3.81	+7.61	32.14	41.70
Egg yolk...........	65.56	11.68	3.84	4.22	+7.54	33.70	43.40

I. Brain Sphingomyelin.[4]

The crude sphingomyelin was prepared according to the directions given in the previous communication. This substance was dissolved in five parts of ligroin and one part of alcohol. Alcohol was then added as long as a precipitate formed. The filtrate was allowed to stand over night at 0°C. and again filtered. The final filtrate was concentrated under diminished pressure and poured into acetone.

The material obtained in this manner was further purified by recrystallization out of a solution in equal parts of pyridine and chloroform. This was carried out in several stages. Five recrystallizations were made at room temperature; these were followed by recrystallizations at 30°, and finally at 37°. The recrystallizations were continued until the substance gave a negative orcin test in the presence of a trace of copper acetate.

Elementary Composition of Sphingomyelin.

I. 0.2835 gm. substance, dried in a vacuum desiccator at the temperature of boiling water, gave 0.0406 gm. $Mg_2P_2O_7$.

0.1890 gm. material required for neutralization 5.1 cc. $\frac{N}{10}$ H_2SO_4.

0.1000 gm. substance gave 0.2430 gm. CO_2, 0.1006 gm. H_2O, and 0.0104 gm. ash.

The optical rotation of the substance dissolved in equal parts of chloroform and methyl alcohol was as follows:

$$[\alpha]_D^{25} = \frac{+0.34° \times 3.6220}{0.5 \times 0.3000} = +8.20°$$

II. 0.3000 gm. substance, dried as Sample I, gave 0.0428 gm. $Mg_2 P_2O_7$.

0.2000 gm. substance required 5.2 cc. $\frac{N}{10}$ H_2SO_4.

0.0996 gm. substance gave 0.2438 gm. CO_2 and 0.1012 gm. H_2O.

The optical rotation in chloroform-methyl alcohol solution was:

$$[\alpha]_D^{25} = \frac{+0.34° \times 3.6198}{0.5 \times 0.3000} = +8.20°$$

III. 0.4000 gm. substance, dried as Sample I, gave 0.0564 gm. $Mg_2P_2O_7$.

0.2000 gm. substance required for neutralization 5.1 cc. $\frac{N}{10}$ H_2SO_4.

0.0990 gm. substance gave 0.2402 gm. CO_2 and 0.1044 gm. H_2O.

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[4] The mode of procedure described for the brain tissue was followed in the work on the other organs.

The optical rotation in chloroform-methyl alcohol solution was:

$$[\alpha]_D^{2.5} = \frac{+0.31^\circ \times 3.6460}{0.5 \ \ \times 0.3000} = +7.53^\circ$$

IV. 0.3000 gm. substance, dried as Sample I, gave 0.0434 gm. $Mg_2P_2O_7$.
0.2000 gm. substance required for neutralization 5.2 cc. $\frac{N}{10}$ H_2SO_4.
0.0990 gm. substance gave 0.2410 gm. CO_2 and 0.0998 gm. H_2O.
The optical rotation in the same solution as the other samples was:

$$[\alpha]_D^{2.5} = \frac{+0.31^\circ \times 3.6404}{0.5 \ \ \times 0.3000} = +7.52^\circ$$

Table of Analysis.

	C	H	N	P
I..................	66.27	11.26	3.78	3.99
II..................	66.81	11.30	3.64	3.97
III..................	66.65	11.59	3.57	3.92
IV	66.45	11.21	3.64	4.02
Average..............	66.59	11.34	3.66	3.98

The variations in the elementary composition of the various samples are slight, especially when the size of the molecule and the physical properties of the substance are taken into consideration.

Methods of Hydrolysis.

Two methods of hydrolysis were employed in the course of the work.

One was described in the earlier paper and was carried out in two stages. In the first the substance was heated with barium hydroxide, in the second with hydrochloric acid. This method will be referred to as the *combined method*.

The second method was carried out in the following way. One part of the substance and fifteen parts by weight of 3 per cent sulphuric acid were heated in sealed tubes for 24 hours at a temperature of 100-105°. From time to time the tubes were agitated. On cooling, the fatty acids and sphingosine sulphate separated as a soft cake which could be readily removed by filtration and washed.

Separation of the Product of Hydrolysis into Three Fractions.

Choline Fraction.—This was contained in the aqueous part of the products of hydrolysis. The analysis of this fraction was carried out in the manner described in the previous paper.

Acid Fraction.—In all experiments the acids were separated from the bases by virtue of the nearly complete insolubility of the barium salts of the higher fatty acids in acetone. The free bases, on the other hand, are soluble in the same solvent.

Sphingosine Fraction.—This was present in the acetone filtrate from the soaps.

For convenience of discussion of the results obtained in this work the mode of procedure in the two types of hydrolysis will be reiterated.

Combined Hydrolysis.—The water-insoluble part of the products of hydrolysis was dissolved in a minimum volume of methyl alcohol to which was added in excess a solution of barium hydroxide in methyl alcohol; to complete the separation of the barium soaps an excess of acetone was added. The precipitate consisted of the soaps, and the filtrate contained the ethyl esters of the fatty acids and the water-insoluble bases.

In order to saponify the esters the acetone solution was concentrated under diminished pressure and the residue dissolved in methyl alcohol. To this solution, barium hydroxide in methyl alcohol was added in excess and the product heated on the water bath with a reflux condenser for 6 hours. From the reaction product the separation of the barium soaps was completed by the addition of a considerable quantity of acetone. To further purify the acid fraction the acids were liberated from the barium soaps, and reconverted into the barium soaps by the barium and acetone procedure.

In order to purify the basic fraction the acetone solution was concentrated *in vacuo*, the residue dissolved in methyl alcohol, and the barium hydroxide and acetone procedure was repeated. As a rule, the entire process was repeated three times. The careful treatment of this fraction was found essential. The presence of impurities, particularly of inorganic nature, interferes considerably with the subsequent reduction of the bases with hydrogen.

Sulphuric Acid Hydrolysis.—The water-insoluble products of hydrolysis under these conditions consisted only of the acids and of sphingosine; there was no need for the stage which aimed to saponify the esters. Hence the reaction product was dissolved in methyl alcohol and treated with barium hydroxide and acetone, as described above.

Quantitative Hydrolysis.—These experiments aimed to determine the proportion of the constituents, and their general elementary composition, without attempting to determine the exact nature of the acids and the bases. Their purpose was to ascertain whether the substances isolated in the course of this work make up the entire molecule of sphingomyelin. This hydrolysis was carried out by means of 3 per cent sulphuric acid. 2 gm. of the substance were used for each experiment.

Analysis of Fatty Acids.

Liberation and Purification of Fatty Acids.—The mode of liberation of the fatty acids and of their purification remained the same in all experiments. The barium soaps were suspended in aqueous 10 per cent hydrochloric acid, and allowed to stand on the water bath over night. On cooling, a solid mass formed consisting of the free acids and some adhering barium chloride. The mass was filtered and taken up in hot acetone, which separated the acids from the mineral impurities. The acetone solution was then concentrated *in vacuo* and the residue was again taken up in acetone. The operation was repeated until the residue completely dissolved in acetone, leaving no filterable residue. The acetone was then removed by distillation and the residue concentrated on the water bath to constant weight.

The fatty acids obtained in this manner served for the determination of the elementary composition and of the molecular weight of the mixed acids. Whenever there was still present a slight mineral impurity, the acids were further purified by transferring them into lead salts. The mode of procedure was the following: The acids were dissolved as rapidly as possible in hot methyl alcohol, and the solution was treated with a slight excess of lead acetate dissolved in methyl alcohol. The addition of a few drops of ammonia completed the precipitation. The lead salts were

washed with water and with acetone, and then suspended in ether and allowed to stand over night. Finally the lead salts were dissolved in hot toluene and decomposed by means of hydrogen sulphide.

Practically all samples of which the molecular weights are recorded were prepared by this procedure.

Separation of the Fatty Acids.—A general method for the separation of fatty acids of sphingomyelin has as yet not been devised. Of the many employed in course of the present work only those will be reported which helped to bring out two points: first, that one of the acids making up the molecule of the phosphatide is lignoceric; and second, that the other is of a lower molecular weight and appears to be a hydroxy acid.

Experiment I.—90.0 gm. of sphingomyelin were hydrolyzed by the combined method. The acids were isolated in two fractions: the first as the barium salts of the unesterified acids; and the second as the barium salts of the saponified esters.

Analysis of the purified acids of the first fraction:

0.1000 gm. substance gave 0.2769 gm. CO_2 and 0.1144 gm. H_2O.

1.0000 " " required for neutralization 6.4 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C.	78.20	75.49
H.	13.20	12.71
Mol. wt.	368	312

Acids of the second fraction:

0.1000 gm. substance gave 0.2772 gm. CO_2 and 0.1158 gm. H_2O.

1.0000 " " required for neutralization 6.4 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C.	78.20	75.59
H.	13.20	12.95
Mol. wt.	368	312

The acids were then combined and repeatedly recrystallized at 25°C. out of a large volume of acetone. Finally the most insoluble fraction had a melting point of 80–82°.[5]

1.0000 gm. of this substance required for neutralization 5.6 cc. $\frac{N}{2}$ alkali; hence mol. wt. = 358.

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[5] All melting points were determined under exactly the same condition; namely, in a sulphuric acid bath provided with a stirring arrangement. The rate of heating was 6 to 7 seconds per 1°C.

Experiment II.—30.0 gm. substance were hydrolyzed in sealed tubes. The fatty acids were immediately transformed into the methyl esters. The esters were distilled under a pressure of 0.3 mm. Two fractions were obtained, the first distilling up to 155°, the second distilling up to 200°.

The esters of the second fraction had a melting point of 56–57°C., and the following composition:

0.1010 gm. substance gave 0.2906 gm. CO_2 and 0.1190 gm. H_2O.

	Calculated for $C_{24}H_{47}O_2CH_3$:	Found:
C	78.53	78.47
H	13.00	13.19

The ester was saponified and the resulting acid had a melting point of 81°C.

Experiment III.—100.0 gm. of sphingomyelin were decomposed by the combined method. The acids were purified by the lead method and fractionated from acetone. The fraction of higher solubility had the following composition:

0.1025 gm. substance gave 0.2758 gm. CO_2 and 0.1096 gm. H_2O.

C = 73.46; H = 11.98.

The substance, again recrystallized out of acetone, had the following composition:

0.1054 gm. substance gave 0.2778 gm. CO_2 and 0.1166 gm. H_2O.

0.3000 " " required 2.0 cc. $\frac{N}{2}$ alkali for neutralization.

The substance had a melting point of 68–69°C.

	Calculated for $C_{18}H_{36}O_3$:	Found:
C	71.92	71.89
H	12.10	12.38
Mol. wt	300	300

The substance was converted into the ethyl ester and the ester recrystallized out of alcohol. The middle fraction had the following composition:

0.0844 gm. substance gave 0.2246 gm. CO_2 and 0.0916 gm. H_2O.

	Calculated for $C_{18}H_{35}O_3C_2H_5$:	Found:
C	73.07	72.58
H	12.27	12.15

This acid possessed the elementary composition and the molecular weight of hydroxystearic acid. However, the low melting point of the acid warned us against hasty conclusions. A corresponding fraction from the kidney sphingomyelin possessed a still lower molecular weight. Hence decision as to the actual composition of the second acid will be deferred until we accumulate more material which will permit further purification of the acid.

Bases.

The mode of procedure in the analysis of the sphingosine fraction differed in the earlier and later experiments. Originally the fraction was treated with alcoholic sulphuric acid to form the sulphates of the bases. These were then reduced with hydrogen gas in the presence of colloidal palladium after Paal. In later experiments it was found more advantageous to reduce the entire fraction prior to the formation of the sulphates. In order to facilitate reduction, the bases were dissolved in ether and washed with water in a separatory funnel. To the ethereal solution of the bases some acetic acid was added and the solution then reduced with hydrogen.

In order to remove the palladium completely the product of reduction was warmed on the water bath with an equal volume of acetone. The filtrate was concentrated to remove all the acetone and acetic acid. The residue was then dissolved in an equal volume of alcohol and an alcoholic solution of sulphuric acid was added until the mixture reacted faintly acid to Congo red. Care must be taken not to overadd sulphuric acid, as the sulphate is quite soluble in an excess of the acid.

Analysis of the bases obtained in this manner gave the following results:

I. Combined method of hydrolysis.
  0.1024 gm. substance gave 0.2772 gm.·CO_2 and 0.0954 gm. H_2O.
  C = 62.37; H = 10.35.

II. Combined method of hydrolysis.
  0.1012 gm. substance gave 0.2336 gm. CO_2 and 0.0970 gm. H_2O.
  C = 62.79; H = 10.80.

A great many samples of similar composition were analyzed, and material of such composition served for the isolation of hydroxyheptadecylamine.

Hydroxyheptadecylamine (*Sphingine*),[6] $C_{17}H_{34}(OH)NH_2$.—This base was obtained by fractional crystallization of the mixed sulphates from a solution of chloroform in amyl alcohol. The first recrystallizations were carried out from a solution in one part of chloroform and three parts of alcohol. The concentration of the chloroform was progressively increased and the final re-

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[6] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1915, xxiv, 67.

crystallizations were made out of equal parts of the solvents, first at room temperature (20–25°C.) and finally at 35°.

The material obtained in this manner consisted of microscopic prismatic needles, and had the macroscopic appearance of glistening scales.

The composition of this crystalline sulphate was not constant for individual samples, nor for successive recrystallizations of the same sample, although a sufficient number gave on analysis values corresponding to the neutral sulphate of sphingine.

I.   0.1020 gm. substance gave 0.2326 gm. CO_2 and 0.1034 gm. H_2O.
     0.2000 "   "   required for neutralization 5.8 cc. $\frac{N}{10}$ acid.
     0.1000 "   "   gave 0.0366 gm. $BaSO_4$.
II. 0.1012 "   "   " 0.2344 gm. CO_2 and 0.0990 gm. H_2O.
     0.2000 "   "   required for neutralization 5.3 cc. $\frac{N}{10}$ acid.
     0.3000 "   "   gave 0.1126 gm. $BaSO_4$.
III. 0.1020 "   "   " 0.2376 gm. CO_2 and 0.1116 gm. H_2O.

	Calculated for $[C_{17}H_{37}NO]_2H_2SO_4$:	I.	Found: II.	III.
C	63.80	63.43	63.43	63.77
H	11.85	11.57	11.01	12.10
N	4.37	4.06	4.06	
S	5.00	5.03	5.15	

. These samples on further recrystallization gave values for carbon varying from 64.5 to 66.0, and for hydrogen from 11.5 to 12.5. However, when converted into the free base they generally had the uniform composition of sphingine.

Free Sphingine.—For the preparation of the free base the sulphate was treated in the same manner as sphingosine sulphate. The base crystallized out of a minimal quantity of petrolic ether in the form of plates resembling cholesterol. The substance had a melting point of 83.5°, and had the following composition:

0.1046 gm. substance gave 0.2844 gm. CO_2 and 0.1280 gm. H_2O.
0.1056 "   "   " 0.2868 "   "   " 0.1238 "   "

	Calculated for $C_{17}H_{37}NO$:	Found:	
C	75.30	74.90	75.35
H	13.65	13.82	13.35

In a 10 per cent solution of sulphuric acid in alcohol the substance had the following rotation:

$$[\alpha]_D^{25} = \frac{-0.34° \times 1.7660}{1 \quad \times 0.1000} = -6.00°$$

Diacetylhydroxyheptadecylamine.—0.6000 gm. of the free base was heated with acetic anhydride for 1 hour under a reflux condenser. On cooling the acetyl derivative crystallized in the form of long needles. The substance was recrystallized out of acetone. It melts at 109.5°C. (corrected).

0.1050 gm. substance gave 0.2730 gm. CO_2 and 0.1108 gm. H_2O.
0.1000 "   "   required for neutralization 2.71 $\frac{N}{10}$ cc. acid.

	Calculated for $C_{17}H_{33}NO(CH_3CO)_2$:	Found:
C	70.90	70.90
H	11.55	11.80
N	3.97	3.79

In a solution in equal parts of chloroform and methyl alcohol the substance gave the following rotation:

$$[\alpha]_D^{25} = \frac{+0.44° \times 2.3234}{1 \quad \times 0.0500} = +20.44°$$

Dihydrosphingosine (Dihydroxyheptadecylamine).—For the identification of sphingosine, use was made of the hydrolysis by means of 3 per cent aqueous sulphuric acid. The basic fraction was reduced with hydrogen in the presence of colloidal palladium. Since the purification by means of repeated recrystallization out of alcohol containing small quantities of sulphuric acid gave unsatisfactory results, it was considered essential to analyze the free base.

Analysis of the sulphate:

0.1040 gm. substance gave 0.2324 gm. CO_2 and 0.1070 gm. H_2O.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$:	Found:
C	60.61	60.94
H	11.38	11.51

The free base was obtained by the process employed in course of this work.

0.1023 gm. substance gave 0.2664 gm. CO_2 and 0.1204 gm. H_2O.

	Calculated for $C_{17}H_{37}NO_2$:	Found:
C	71.07	71.02
H	12.89	13.17

Hydroxyheptadecylamine from Cerebrin.—80.0 gm. of cerebrin, corresponding to Thierfelder's cerebron, were hydrolyzed by the

combined method, and the mode of procedure employed in the hydrolysis of sphingomyelin was followed closely. The free base obtained in this manner had the physical properties of sphingine and the following composition:

0.1042 gm. substance gave 0.2874 gm. CO_2 and 0.1232 gm. H_2O.

	Calculated for $C_{17}H_{37}NO$:	Found:
C	75.30	75.12
H	13.65	13.23

Choline.—Choline was isolated from the aqueous part of the product of hydrolysis. When the hydrolysis was accomplished by means of aqueous sulphuric acid, the aqueous filtrate was treated with barium carbonate and the filtrate from barium sulphate was concentrated to dryness under diminished pressure. The residue was taken up in alcohol, filtered, and concentrated, and the operation was repeated several times. The final product was acidulated with hydrochloric acid and treated with an alcoholic solution of platinic chloride. The platinic salt was recrystallized out of water.

When barium hydroxide was used for hydrolysis the aqueous part of the product of hydrolysis was saturated with carbon dioxide gas, and the filtrate from barium carbonate was used for the preparation of the chloroplatinic salt of choline. The solution apparently contained no other bases than choline, since on treatment with nitrous acid in Van Slyke's apparatus it did not give off nitrogen gas.

0.1018 gm. substance gave 0.0732 gm. CO_2, 0.0430 gm. H_2O, and 0.0316 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.60
H	4.58	4.69
Pt	31.65	31.05

Quantitative Hydrolysis.

In order to obtain an estimate of the fatty acids and of sphingosine in sphingomyelin, hydrolysis was carried out on 2.0 gm. of the substance in a sealed tube with a 3 per cent sulphuric

acid solution. The mode of procedure was the same as that carried out on larger quantities of material.

The empirical data obtained in this work will be compared with the theoretical for the monophosphatide containing lignoceric acid in its molecule.

	Calculated for $C_{44}H_{88}N_2O_7P$:	Found:
Sphingosine	35.56	34.10
Acids	40.58	43.00

The crude sphingosine fraction contained 4.5 per cent nitrogen. It was transformed into a sulphate of the following composition:

0.1012 gm. substance gave 0.2218 gm. CO_2 and 0.0928 gm. H_2O.

	Calculated for $(C_{17}H_{35}NO_2)_2H_2SO_4$:	Found:
C	61.08	60.97
H	10.78	10.47

Analysis of the acid fraction:

0.1022 gm. substance gave 0.2876 gm. CO_2 and 0.1188 gm. H_2O.

1.0000 " " was titrated with $\frac{N}{2}$ alkali, and required for neutralization 6.3 cc.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	76.75
H	13.20	13.00
Mol. wt	368	317

II. Beef Kidney Sphingomyelin.

A phosphatide of this group was first discovered in the extracts from beef kidney by E. K. Dunham,[7] and was named by him carnaubon. Later MacLean[8] questioned the correctness of Dunham's views by showing that carnaubon could be further purified, and still later Rosenheim and MacLean[9] have demonstrated that carnaubon contained an admixture of galactosides, and that the acid assumed by Dunham to be carnaubic had the physical properties of lignoceric acid. However, Rosenheim and MacLean have not isolated the pure sphingomyelin.

[7] Dunham, E. K., Proc. Soc. Exper. Biol. and Med., 1904-05, ii, 63; Jour. Biol. Chem., 1908, iv, 297. Dunham, E. K., and Jacobson, C. A., Z. f. physiol. Chem., 1910, lxiv, 302.

[8] MacLean, H., Biochem. Jour., 1912, vi, 333.

[9] Rosenheim, O., and MacLean, H., Biochem. Jour., 1915, ix, 103.

The present investigation was in progress at the time of the publication of their results.

Preparation and Composition.—All the details found necessary for the preparation of brain sphingomyelin were applied for the isolation of the substance from the kidney tissue.   Several samples were analyzed and all seemed to contain a slightly lower proportion of carbon.   This was possibly due to the fact that the material from the kidney contained a higher proportion of the phosphatide with the fatty acid of a molecular weight lower than that of lignoceric.

I. 0.1032 gm. substance gave 0.2412 gm. CO_2 and 0.1052 gm. H_2O.

   0.3000 "   "   required for neutralization 7.5 cc. $\frac{N}{10}$ acid.

   0.4000 "   "   gave 0.0548 gm. $Mg_2P_2O_7$.

II. 0.0990 "   "   "   0.2330 gm. CO_2 and 0.1042 gm. H_2O.

   0.2000 "   "   required for neutralization 4.75 cc. $\frac{N}{10}$ acid.

   0.3000 "   "   gave 0.0404 gm. $Mg_2P_2O_7$.

	C	H	N	P
I	64.80	11.41	3.50	3.82
II	64.53	11.83	3.52	3.96

In a solution of methyl alcohol and chloroform Sample I gave the following optical rotation:

$$[\alpha]_D^{16} = \frac{+0.36° \times 3.6510}{0.5 \quad \times 0.3000} = +8.73°$$

Quantitative Hydrolysis.

2.0 gm. of Sample I, hydrolyzed for 24 hours in a sealed tube with 3 per cent sulphuric acid, gave 32.1 per cent of its weight in the sphingosine fraction and 49.0 per cent in the fraction of the fatty acid.

Choline Fraction.—This fraction was analyzed in the same manner as the corresponding fraction from brain sphingomyelin.   The base was identified as the salt of chloroplatinic acid.   The salt was recrystallized out of water.

0.1048 gm. substance gave 0.0752 gm. CO_2, 0.0434 gm. H_2O, and 0.0334 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.58
H	4.58	4.63
Pt	31.65	31.87

Acid Fraction.—Here also final results on the separation of the two acids have not yet been obtained.   The mixed acid showed approximately the same composition and molecular weight as the corresponding material from the brain sphingomyelin.

Experiment I.—Acid hydrolysis. The acid fraction was purified through the lead salts.

I. 0.1020 gm. substance gave 0.2830 gm. CO_2 and 0.1135 gm. H_2O.
II. 0.1014 "   "   " 0.2846 "   "   " 0.1128 "   "
    0.4062 " mixed acids required for neutralization 2.56 cc. $\frac{N}{2}$ alkali.

	Calculated for $C_{24}H_{48}O_2$:	Found: I.	II.
C	78.20	75.96	76.53
H	13.20	12.49	12.45
Mol. wt	368	312	315

Experiment II.—The most convenient method for obtaining lignoceric acid was through the process of alcoholysis. 9.0 gm. of sphingomyelin in a solution of 4.0 cc. of sulphuric acid in 200 cc. of methyl alcohol were heated under a reflux condenser on the water bath for 6 hours. On cooling an ester separated out in bright scales. Recrystallized out of acetone, the substance contained: C, 74.11, and H, 12.13. The substance contained 1.17 per cent of ash. It was distilled at a pressure of 0.4 mm. The temperature of distillation was allowed to rise to 200°C. The distillate was recrystallized out of acetone. The substance had a melting point of 56–57° and the following composition:

0.1020 gm. substance gave 0.2934 gm. CO_2 and 0.1200 gm. H_2O.

	Calculated for $C_{24}H_{47}O_2CH_3$:	Found:
C	78.53	78.44
H	13.09	13.17

The acid of the lower molecular weight has not yet been isolated in a sufficient degree of purity. The best results were obtained by fractionation from acetone. In two experiments a substance was obtained of apparently the same composition and molecular weight. But here again decision regarding its true composition will have to be deferred until a larger quantity of the acid is on hand. In both experiments the acids were purified through the lead salts prior to fractionation. The two results will be described under Experiment III.

Experiment III.—The mixed acids were recrystallized out of acetone at 0°, the mother liquor was concentrated and again taken up in hot acetone. The final substance had the following composition

0.0952 gm. substance gave 0.2558 gm. CO_2 and 0.1152 gm. H_2O.
C = 69.76; H = 10.65.

A second sample, obtained in the same manner, had the following composition:

0.1042 gm. substance gave 0.2670 gm. CO_2 and 0.1032 gm. H_2O.
C = 69.88; H = 11.09.

Sphingosine Fraction.—The mixed bases obtained by the combined method of hydrolysis, following exactly the mode of procedure described above, gave results approximating those obtained on the brain sphingomyelin. The product consisted of long needles.

0.1046 gm. substance gave 0.2400 gm. CO_2 and 0.1092 gm. H_2O.
C = 62.79; H = 11.69.

This substance was further fractionated from amyl alcohol and chloroform and finally transformed into the free base. This had the appearance of sphingine and the following composition:

0.1030 gm. substance gave 0.2756 gm. CO_2 and 0.1222 gm. H_2O.

	Calculated for $C_{17}H_{37}NO$:	Calculated for $C_{17}H_{37}NO_2$:	Found:
C..............................	75.30	71.07	73.12
H..............................	13.65	12.89	13.30

Pure sphingosine was more accessible through the sulphuric acid hydrolysis. The substance was reduced with hydrogen prior to conversion into the sulphate.

I. 0.0992 gm. substance gave 0.2158 gm. CO_2 and 0.1042 gm. H_2O.
  0.1410   "   "   required for neutralization 4.25 cc. $\frac{N}{10}$ acid.
II. 0.1048   "   "   gave 0.2340 gm. CO_2 and 0.1032 gm. H_2O.
  0.1500   "   "   required for neutralization 4.34 cc. $\frac{N}{10}$ acid.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$:	Found: I.	II.
C..............................	60.61	60.67	60.90
H..............................	11.38	11.35	11.02
N..............................	4.16	4.23	4.05

Lignoceryl Sphingine.

The substance was obtained in the course of a combined hydrolysis of 100 gm. of Sample I (page 84). The basic fraction was reduced with hydrogen in the presence of palladium after Paal.

The reaction product was warmed on the water bath and filtered. The filtrate was concentrated nearly to dryness on the water bath. The residue was dissolved in 400 cc. of hot alcohol and filtered hot. While the solution was still warm a crystalline precipitate formed. This was immediately filtered, and recrystallized out of alcohol. It crystallized in the form of rosettes composed of needles.

The substance gave no nitrogen gas on treatment with nitrous acid according to Van Slyke.

0.1018 gm. substance gave 0.2874 gm. CO_2 and 0.1173 gm. H_2O.
0.1473 "   "   neutralized 2.72 cc. $\frac{N}{10}$ acid.

	Calculated for $C_{17}H_{34}(OH)NH.C_{18}H_{47}O$:	Found:
C	79.39	79.60
H	13.36	13.32
N	2.26	2.59

Lack of material prevented further work on this substance.

III. Liver Sphingomyelin.

No phosphatide of this type has been previously obtained from this organ. The substance here analyzed was prepared in the usual way. The crude sphingomyelin required a great many recrystallizations from pyridine and chloroform. Finally it reacted towards orcin in the same manner as sphingomyelin of other origin.

0.1067 gm. substance gave 0.2500 gm. CO_2 and 0.1088 gm. H_2O.
0.3000 "   "   required for neutralization 7.3 cc. $\frac{N}{10}$ acid.
0.4000 "   "   gave 0.0548 gm. $Mg_2P_2O_7$.

C	H	N	P
64.47	11.57	3.41	3.81

In a solution of equal parts of methyl alcohol and chloroform the substance had the following rotation:

$$[\alpha]_D^{32} = \frac{+0.31° \times 3.6490}{0.5 \times 0.3000} = +7.61°$$

Quantitative Hydrolysis.

2.0 gm. substance, hydrolyzed with 3 per cent H_2SO_4, gave 32.14 per cent of its weight in the form of sphingosine and 41.70 per cent in the form of acids.

	Calculated for $C_{48}H_{93}N_2O_7P$:	Found:
Sphingosine	35.56	32.14
Acids	40.58	41.70

Choline.—This base was obtained in the usual way. The salt of chloroplatinic acid was recrystallized out of water.

0.1000 gm. substance gave 0.0718 gm. CO_2, 0.0428 gm. H_2O, and 0.0312 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.58
H	4.58	4.79
Pt	31.65	31.20

Sphingosine.—The substance was obtained in the usual way. It was analyzed in the form of the sulphate.

0.1075 gm. substance gave 0.2282 gm. CO_2 and 0.0952 gm. H_2O.

	Calculated for $(C_{17}H_{35}NO_2)_2H_2SO_4$:	Found:
C	61.08	60.78
H	10.78	10.40

Acids.—The mixed acids were obtained by hydrolysis with 3 per cent sulphuric acid in sealed tubes and had the following composition:

0.1075 gm. substance gave 0.2956 gm. CO_2 and 0.1206 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	75.00
H	13.20	12.60

IV. Egg Yolk Sphingomyelin.

A phosphatide approximating in composition that of Thudichum's sphingomyelin was described by Stern and Thierfelder.[10] The material described here was prepared by the same mode of procedure used in the work on sphingomyelin from other sources and had the same microscopic appearance. It gave a negative test on boiling with orcin in the presence of copper salts.

0.0993 gm. substance gave 0.2354 gm CO_2 and 0.1030 gm. H_2O.
0.4000 "   "   gave 0.0606 gm. $Mg_2P_2O_7$.
0.3000 "   "   required for neutralization 8.3 cc. $\frac{N}{10}$ acid.

C	H	N	P
65.00	11.68	3.84	4.22

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[10] Stern, M., and Thierfelder, H., *Z. f. physiol. Chem.*, 1907, liii, 370.

In a solution of equal parts of methyl alcohol and chloroform the substance had the following rotation:

$$[\alpha]_D^{25} = \frac{+0.31° \times 3.6500}{0.5 \times 0.3000} = +7.54°$$

Quantitative Hydrolysis.

2 0 gm. substance, hydrolyzed with 3 per cent sulphuric acid, gave in the acid fraction 43.14 per cent of its weight and 33.70 per cent in the sphingosine fraction.

	Calculated for $C_{46}H_{93}N_2O_7P$:	Found:
Sphingosine	35.56	33.70
Acids	40.58	43.14

Choline.—This was obtained in the usual way and recrystallized out of water.

0.1030 gm. substance gave 0.0734 gm. CO_2, 0.0460 gm. H_2O, and 0.0324 gm. Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$:	Found:
C	19.48	19.13
H	4.58	4.88
Pt	31.65	31.46

Sphingosine Fraction.—This was obtained in the usual way and reduced to dihydrosphingosine. The value obtained for the carbon slightly exceeds the theoretical requirement, and the evidence is accepted as satisfactory only in the light of the experience gained with the substance obtained from the brain and from the kidneys.

0.1039 gm. substance gave 0.2348 gm. CO_2 and 0.1054 gm. H_2O.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$:	Found:
C	60.61	61.75
H	11.38	11.37

Analysis of Mixed Acids.—The acids were obtained by hydrolysis with 3 per cent sulphuric acid and had the same composition as the acids obtained from sphingomyelin originating from other sources.

0.1070 gm. substance gave 0.2914 gm. CO_2 and 0.1266 gm. H_2O.

	Calculated for $C_{24}H_{48}O_2$:	Found:
C	78.20	75.29
H	13.20	12.24

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THE MODE OF ACTION OF THE OXIDASES.[1]

By HERBERT H. BUNZELL.

(From the Bureau of Plant Industry, United States Department of Agriculture, Washington.)

INTRODUCTION.

CORRECTION.

On page 57, Vol. XXIV, No. 1, January, 1916, in the last line, for
— *2.89°* read —*0.289°*.

of certain compounds, and the reactions exhibited by means of
this mechanism may equally well be explained in other ways,
the writer has adopted the term "oxidase activity" rather than
"concentration of oxidases" to denote the factors responsible for
the complex of reactions based on oxygen absorption in the
presence of plant juices or tissues. In the writer's mind the
concept has for some time existed in the broader form just stated,
although in his papers the terms "concentration of oxidases"
and "oxidase activity" have been used interchangeably. To
express the activity of a plant juice in this respect, the following
unit has been accepted provisionally: "An oxidase solution of
such strength that 1 liter of it will be capable of bringing about
the consumption by the reagent used of the equivalent of 1 gm.

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THE MODE OF ACTION OF THE OXIDASES.[1]

By HERBERT H. BUNZELL.

*(From the Bureau of Plant Industry, United States Department of
Agriculture, Washington.)*

INTRODUCTION.

This paper deals only with such peculiar conditions existing in
fresh plant tissues as are capable of bringing about the oxidation
by means of atmospheric oxygen of certain aromatic compounds
closely related to phenol, usually provided with two or more
OH or CH_3 groups. Inasmuch as these peculiar conditions
existing in plants were hitherto and even now are generally
explained by assuming the existence of certain chemical com-
pounds of catalytic properties called oxidases, the efficiency of
the factors underlying the conditions referred to seems in general
correlated with the concentration of the oxidases present in the
tissue. Since there is no convincing evidence to the effect that
the oxygen transfer mechanism is based purely on the presence
of certain compounds, and the reactions exhibited by means of
this mechanism may equally well be explained in other ways,
the writer has adopted the term "oxidase activity" rather than
"concentration of oxidases" to denote the factors responsible for
the complex of reactions based on oxygen absorption in the
presence of plant juices or tissues. In the writer's mind the
concept has for some time existed in the broader form just stated,
although in his papers the terms "concentration of oxidases"
and "oxidase activity" have been used interchangeably. To
express the activity of a plant juice in this respect, the following
unit has been accepted provisionally: "An oxidase solution of
such strength that 1 liter of it will be capable of bringing about
the consumption by the reagent used of the equivalent of 1 gm.

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of hydrogen; *i.e.*, of 8 gm. of oxygen."[2] If instead of a plant juice or a modified plant juice we work with dried plant tissue, the moisture content has to be taken into account in the calculation.

The aromatic compounds that have been used as oxidase reagents are numerous.[3] It has been shown for a number of them, and is presumably true of all, that they undergo a spontaneous oxidation in aqueous solution in the presence of oxygen. The rate of oxidation may be greatly accelerated by means of alkalies, heavy metal ions, and oxidase-active animal or plant tissues or extracts of the same. Such animal or plant tissues or extracts would seem to be, therefore, catalytic agents. In this paper only increased oxygen absorption due to the presence of plant juices or tissues will be discussed.

Numerous experiments embodying the study of the rate of oxidation of some of the oxidase reagents in the presence as well as in the absence of plant juices have been made according to the methods developed in this laboratory.[4] From all these experiments the fact stands out that the accelerating action of the plant material is ended after a definite period. This period ranges within wide limits, according to the experimental conditions, nature of the plant material and reagent used—in our experiments from ½ hour to 3 or 4 hours. To illustrate this point the following experiments were selected at random.

[2] Bunzell, H. H., The Measurement of the Oxidase Content of Plant Juices, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 238*, 1912, 40.

[3] Kastle, J. H., The Oxidases and Other Oxygen-Catalysts Concerned in the Biological Oxidases, *Bull. Hyg. Lab., U. S. P. H., 59*, 1910. Clark, E. D., The Plant Oxidases, New York, 1910. Begeman, O. H. K., Beiträge zur Kenntnis pflanzlicher Oxydationsfermente, *Arch. f. d. ges. Physiol.*, 1915, clxi, 45.

[4] Bunzell, Ein neuer Apparat zur Bestimmung von Oxydasen in Gewebesäften, *Z. f. biol. Tech. u. Meth.*, 1912, ii, 307; The Measurement of the Oxidase Content of Plant Juices, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 238*, 1912; A Biochemical Study of the Curly-Top of Sugar Beets, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 277*, 1913; Quantitative Oxidase Measurements, Orig. Com., *VIIIth Inter. Cong. Applied Chem.*, 1912, xix, 37; Biological Oxidizability and Chemical Constitution, *Jour. Biol. Chem.*, 1914, xvii, p. xxxvi; A Simplified and Inexpensive Oxidase Apparatus, *Jour. Biol. Chem.*, 1914, xvii, 409; Oxidases in Healthy and in Curly-Dwarf Potatoes, *Jour. Agric. Research*, 1914, ii, 373; On Alfalfa Laccase, *Jour. Biol. Chem.*, 1915, xx, 697.

TABLE I.

Material: Dried and Finely Powdered Potato Peels. 0.05 Gm.
Reagent: Pyrogallol, 0.01 Gm.
Small Apparatus.* 6 Cc. H_2O.

Time.	Reading.†
10.30	0
10.45	−1.50
11.00	−1.45
11.15	−1.35
11.30	−1.35
11.45	−1.35
12.00	−1.35

TABLE II.

Material: Dried and Finely Powdered Tulip Tree Buds. 0.01 Gm.
Reagent: Pyrocatechol, 0.01 Gm.
Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
10.45	0
11.00	−1.15
11.15	−2.20
11.30	−2.30
11.45	−2.70
1.00	−3.00
1.30	−3.00

* Bunzell, Jour. Biol. Chem., 1914, xvii, 409.
† All readings are expressed in terms of centimeters of mercury.

TABLE III.

Material: Tulip Tree Leaves. 0.10 Gm.
Reagent: Phlorhizin, 0.01 Gm.
Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
11.00	0
11.15	−1.40
11.30	−2.25
11.45	−3.00
12.00	−3.40
12.30	−3.80
12.45	−4.00
1.00	−4.00

TABLE IV.

Material: Dried and Finely Powdered Spinach Leaves. 0.10 Gm.
Reagent: p-Cresol, 0.02 Gm. Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
10.15	0
10.30	−0.20
10.45	−0.60
11.00	−0.80
11.15	−1.20
11.30	−1.40
11.45	−1.45
12.00	−1.65
12.15	−1.65
12.30	−1.65

TABLE V.

Material: Dried and Finely Powdered Spinach Roots. 0.025 Gm.
Reagent: Tyrosine, 0.01 Gm. Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
10.00	0
10.45	−1.05
11.00	−1.20
11.30	−1.20
11.45	−1.20
12.30	−1.15
12.45	−1.20

TABLE VI.

Material: Dried and Finely Powdered Canna Stems. 0.02 Gm.
Reagent: Guaiacol, 0.02 Gm. Small Apparatus. 6 Cc. H_2O.

Time.	Reading.
2.15	0
2.30	−1.30
2.45	−1.50
3.00	−1.65
3.15	−1.70
3.30	−1.90
3.45	−1.90

Control experiments with aqueous solutions of the oxidizable materials used in these experiments show no measurable oxygen absorption. This behavior, of course, does not mean that they are not oxidized under the conditions of the control experiments, but that the rate of oxidation is insignificant compared with that taking place in the presence of plant materials.

There are a number of causes which might account for the rather abrupt ending of the influence of the plant material. The writer set out to determine whether the standstill which is apparent in all the oxidase reactions studied is due to the exhaustion of the agents responsible for the oxidase activity or to some inhibitory influence arising in the course of the reaction. The following inhibitory factors were considered.

1. For reasons of technique the experiments were mostly carried out at 37°C., a temperature which may be somewhat destructive to the agencies involved.

2. Shaking is known to have a deleterious influence on some enzymes and may also affect the agencies involved.

3. The reagents used may have an inhibiting effect on the reaction.

4. The products formed in the course of the oxidation may have a retarding and even an inhibiting influence on the reaction, by driving the reaction in the opposite direction, by combining with the active substances present, or by unfavorably changing the reaction of the medium.

5. The available oxidizable material may be used up.

6. The available oxygen may be consumed or its diminished pressure may have a retarding effect.

Experiments were therefore planned to determine the influence of these factors.

EXPERIMENTAL.

1. Effect of Different Temperatures on the Measure of Oxidase Activity.

The results in Table VII indicate that during shaking at the higher temperatures a part of the oxidase activity is destroyed. In general there is a marked diminution in activity as the temperature rises to 47°C.; below that the effect is not so marked

TABLE VII.

Influence of Different Temperatures on Oxygen Absorption. Rate of Shaking: 5 Excursions in 3.2 Seconds. Material: Finely Powdered Dried Potato Peels. In the Case of Solids, 0.01 Gm. Was Used; in the Case of Liquids, Two Drops.

	Pyrogallol.	Pyro-catechol.	Hydro-quinone.	Phlorhizin.	Guaiacol.	Tyrosine.	m-Cresol.	p-Cresol.	Eugenol.	Iso-eugenol.
	Gm. of material used.									
	0.05	0.02	0.02	0.02	0.05	0.10	0.05	0.01	0.10	0.10
°C.	Absorption.									
26.5	1.10	1.25	2.65	1.60	1.00	1.30	2.20	4.40	0	0.20
31.0	1.40	1.35	2.50	2.00	1.70	1.60	2.10	Leak	0.20	0.60
37.5	0.90	0.90	1.25	1.20	1.70	1.40	2.50	3.40	0.10	0.40
47.4	1.05	0.90	0.90	0.90	1.50	1.05	1.60	Leak	0.20	0.80

and might well be only apparent, due to the experimental fluctuations. On account of these fluctuations it did not seem necessary to reduce the manometer readings to a uniform temperature. The temperature correction would amount to only 3 per cent for a difference in temperature of 10°C., or less than 1 mm. in an absorption corresponding to 3 cm. of mercury.

Even at a temperature as high as 47.4°C. there is still considerable activity. The rather high temperature, therefore, (37°C.) at which most of the work so far has been carried out, probably has a retarding but not an inhibiting effect on the oxidase activity of the plant tissues used in this work.

2. Effect of the Rate of Agitation on the Measure of Oxidase Activity.

From Table VIII it is apparent that the rate of shaking within the limits of these experiments has no marked influence on the final results. The important effects of agitation, namely, the saturation of the solution with oxygen from the air and the distribution of the active plant tissue particles, can be accomplished by shaking with 15 excursions per minute just as well as with 150 excursions per minute.

TABLE VIII.

Influence of the Rate of Agitation on the Oxygen Absorption.* Temperature: 37.2°C. Material: Finely Powdered Dried Potato Peels. In the Case of Solids, 0.01 Gm. Was Used; in the Case of Liquids, Two Drops.

	Pyrogallol.	Pyro-catechol.	Hydro-quinone.	Phlorhizin.	Guaiacol.	Tyrosine.	m-Cresol.	p-Cresol.	Eugenol.	Iso-eugenol.
Gm. of material used......	0.05	0.02	0.02	0.02	0.05	0.10	0.05	0.01	0.10	0.10
5 excursions in 2°C.......	1.20	1.30	2.40	1.70	1.30	1.60	2.10	3.75	0.05	0.10
5 " " 3.2°C.....	1.10	1.25	2.65	1.60	1.00	1.30	2.20	4.40	0	0.20
5 " " 5.4°C......	1.00	1.20	2.20	1.40	1.00	1.75	2.25	4.00	0	0
15 " " 10 sec.once every min.............	1.10	1.30	1.70	1.80	1.20	1.60	2.30	3.40	0.20	0.60

* In this connection the results of Appleman are interesting. Using pyrogallol as reagent and potato juice as plant material, he found that by mild agitation at long intervals the oxygen absorption can be made to spread over several days. (Appleman, C. O., *Maryland Agricultural Experiment Station*, Bull. 183, 1914, 193.)

3. Inhibiting Effect of the Reagents Used on the Measure of Oxidase Activity.

In order to determine what effect is produced on the end-results by an excess of the reagents, it was necessary first to establish what constitutes such an excess. Table IX presents results which were obtained by working with fixed quantities of plant material and varying quantities of reagents.

These results show that *p*-cresol when used in quantities of more than three drops (0.036 gm.) in 6 cc. of liquid retarded the action. There also seems to be a slight retardation in the case of pyrogallol when more than 0.015 gm. was used. For the particular sample of potato material used, 0.01 to 0.015 gm. of the solid reagents seems to be a desirable quantity to use; in the case of the three liquids the situation is less simple; to obtain the maximum result, *p*-cresol would seem to be used best in quantities of two drops (0.024 gm.); *m*-cresol, three to four drops (0.036 to 0.048 gm.); and guaiacol in quantities of four drops (0.056 gm.). These ratios, however, would probably vary according to the particular plant material used and the working conditions.

There seems to be, therefore, no marked retardation due to most of the reagents present, even if they are used in considerable excess.

TABLE IX.

Effect on the End-Readings of the Reagents Used. Temperature: 37°C. Material: Finely Powdered Dried Potato Peels. The Quantity of Material Used in Each Case is Given in the First Perpendicular Column with the Name of the Reagent.

Gm. of reagent.	0.02	0.02	0.015	0.015	0.01	0.01	0.005	0.005	0.0025	0.0025
Pyrocatechol. 0.02 gm. potato powder	1.35	1.50	1.40	1.30	1.20	1.30	1.20	1.10	1.00	Lost.
Phlorhizin. 0.02 gm. potato powder	1.60	2.20	2.00	Lost	1.40	1.60	0.80	1.00	0.20	0.20
Hydroquinone. 0.02 gm. potato powder	2.15	2.00	2.00	2.30	2.00	2.10	1.90	2.10	1.60	1.60
Pyrogallol. 0.05 gm. potato powder	1.15	1.25	1.40	1.50	1.25	1.50	1.60	1.50	1.05	1.00
Tyrosine. 0.10 gm. potato powder	1.40	1.40	1.50	1.50	1.20	1.25	1.05	1.35	0.80	1.05
Quantity of reagent (drops).	5*	5	4	4	3	3	2	2	1	1
Guaiacol. 0.05 gm. potato powder	1.90	2.05	1.90	1.90	1.25	1.40	0.90	1.00	0.50	0.45
m-Cresol. 0.05 gm. potato powder	2.60	2.40	2.55	2.30	2.00	2.00	1.60	1.80	1.10	1.10
p-Cresol. 0.01 gm. potato powder	2.60	2.40	3.00	3.70	3.70	3.90	3.85	3.80	3.30	3.30

* The pipette used for all the experiments was calibrated and it was found that each drop of guaiacol weighed 0.014 gm.; each drop of m- and of p-cresol, 0.012 gm.

4. Effect of Products Formed in the Course of the Reaction on the Measure of Oxidase Activity.

In order to determine whether the products formed are responsible for the cessation of oxygen absorption, the reaction was allowed to come to an end as usual with the ten reagents used and

then once more the same quantities of the plant material were added as in the beginning, and the shaking was continued. The final results are given in Table X.

TABLE X.

Influence of the Products Formed in the Course of the Reaction on the Oxygen Absorption. Temperature: Room. Material: Finely Powdered Dried Potato Peels. In the Case of Solids, 0.01 Gm. Was Used; in the Case of Liquids, Two Drops.

	Pyrogallol.	Pyro-catechol.	Hydro-quinone.	Phlorhizin.	Guaiacol.	Tyrosine.	m-Cresol.	p-Cresol.	Eugenol.	Iso-eugenol.
Gm. of material used........	0.05	0.02	0.02	0.02	0.05	0.10	0.05	0.01	0.10	0.10
First set of results..........	1.40	1.35	2.50	2.00	1.70	1.60	2.10	Leak	0.20	0.60
After addition of more powder in the same quantities as before......	1.40	0.45	1.40	1.75	3.60	3.05	4.30	2.00	0.10	0.50

It is evident that the activity of the plant powder is not paralyzed by the products formed in the course of the reaction. While in the case of some reagents the final result is somewhat reduced, it is augmented in others, unaffected in a few, but completely inhibited in none.

5. Consumption of the Available Oxidizable Material.

Table X gives evidence that the complete utilization of the oxidizable material is not the reason for the cessation of the reaction. As the second row of figures shows, absorption goes on after the addition of fresh plant material without the addition of further quantities of reagents.

6. Consumption of Available Oxygen and the Retarding Effect on the Results of the Diminished Partial Pressure of the Oxygen Present.

It seems improbable that in any one experiment the total available quantity of oxygen should be consumed. If we consider one-fifth of the gas in the apparatus as oxygen and assume

that the change of pressure is entirely due to absorption of oxygen, the extreme variation of pressure as registered by the attached manometer, *i.e.*, 8 cm., would represent the absorption of only about one-half of the oxygen present. If, however, gases are produced in the process, the same change in pressure would correspond to a greater oxygen absorption. To prove conclusively that the consumption of oxygen is not the limiting factor, several series of experiments were carried out with varying quantities of the plant material and different reagents.

TABLE XI.

Results Obtained with Varying Quantities of Plant Material. Temperature: 37°C. Material: Finely Powdered Dried Potato Peels.

Reagent.											
Pyrocatechol 0.02 gm.	Gm. of material used....	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05	0.05
	Reading........	0.65	0.65	1.20	1.30	1.80	1.90	2.70	2.80	3.00	3.10
Pyrogallol 0.015 gm.	Gm. of material used....	0.02	0.02	0.04	0.04	0.06	0.06	0.08	0.08	0.10	0.10
	Reading.......	0.10	0.10	0.50	0.50	1.00	1.00	1.80	1.80	2.00	2.00
Hydroquinone 0.015 gm.	Gm. of material used....	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05	0.05
	Reading........	1.00	1.10	0.40	1.90	3.80	3.20	4.70	4.50	5.50	5.90
Phlorhizin 0.02 gm.	Gm. of material used....	0.01	0.01	0.02	0.02	0.03	0.03	0.04	0.04	0.05	0.05
	Reading........	1.00	1.20	2.00	1.90	2.60	3.10	3.40	3.90	4.20	4.00
Tyrosine 0.015 gm.	Gm. of material used....	0.05	0.05	0.10	0.10	0.15	0.15	0.20	0.20	0.25	0.25
	Reading........	0.50	0.60	1.40	1.15	1.90	2.00	2.50	2.70	3.00	3.00

DISCUSSION.

As these figures show, the final results for each reagent are practically proportional to the quantity of active material used. The deviations from an absolute and direct proportionality lie within the limits of error of experimentation. The definite termination of the reactions when comparatively small quantities of oxidase material were used is not due, therefore, to the con-

sumption of the oxygen present or to its diminished partial pressure, but to complete utilization of the plant material.

The termination of the oxidation of the artificial chromogens under discussion, therefore, is due to the exhaustion of the activity of the plant material. If we wish to consider the activity of the plant juices or plant tissues responsible for the oxygen transfer or catalytic action, we may say that the compounds or the mechanism responsible for the process have become destroyed or injured and have thereby lost their power to act catalytically.

The writer does not contend that the factors mentioned and discussed in the foregoing pages are without influence on the end-result. In many instances they seem to have a decided influence, usually retarding, though sometimes stimulating, but they are not either individually or collectively the cause for the sharp end-point obtained in the course of the oxygen absorption. Even if the factors stated were responsible for the existence of an end-point, we should be justified, for lack of better methods, in using as indicator of the oxidase activity the figures obtained by means of the measurement of the oxygen absorption, because the figures obtained would still be comparable. The greater the original activity, the greater we should expect the final result to be, for this would be a result of the original activity and a number of destructive agencies which would be fairly uniform in all cases.

There is also to be considered the presence of natural chromogens in the material used, which in the presence of the oxidizing enzymes would absorb an appreciable quantity of oxygen. That such natural chromogens are widely existent in many kinds of plant materials, and perhaps in all, is well known. As a concrete example it may be stated that the chromogens in 0.10 gm. of finely powdered tulip tree buds (dried *in vacuo*) brought about in the small apparatus[5] a diminution of pressure of 0.50 to 0.80 cm. These chromogens seem to be found, to some extent at least, in the juice pressed out of the leaves. In the case of tomatoes, with which many experiments were tried, 2 cc. of leaf juice brought about in the small apparatus a diminution of pressure of 1.10 to 1.60 cm. of mercury. Tobacco leaf juice caused a reduction of

[5] Bunzell, A Simplified and Inexpensive Oxidase Apparatus, *Jour. Biol. Chem.*, 1914, xvii, 409.

pressure of 1.75 cm. of mercury. The chromogens, in the case of the tulip tree buds at least, are oxidized by the aid of oxidases. After the powder was boiled in water for 1 minute it was deprived of the power to oxidize either the reagents here used or the natural chromogens. Dried leaf powder of the tulip tree seems to have a chromogen content of about twice that of the buds, as evidenced by the oxygen absorption.

When both oxidase reagent and chromogen are present, with excessive oxidase activity and oxygen, the result (in the case of tomato leaves) is by no means a simple summation of the oxygen absorption of the two. The oxidase activity seems to divide itself between the artificial and the natural chromogens, presumably entering into some preliminary stage with both of them. In this way combination with the artificial chromogen seems to take place, whether the latter is markedly oxidized or not. Presence of a scarcely oxidizable chromogen may in this way greatly depress the oxidation of a natural chromogen. The presence of the reagent pyrocatechol, for example, seems to prevent the oxidation of the chromogen. This phenomenon is illustrated by the following experiment. In parallel experiments diluted tomato juice alone, as well as the same plus pyrocatechol, were shaken and the oxygen absorption was determined in the usual way. The details of the experiment are described in Table XII.

TABLE XII.

Oxidation of Chromogen in Tomato Juice* + Oxidation of Chromogen + Oxidase Reagent (Pyrogallol) Combined. Temperature: 37.2°C. Rate of Shaking: 5 Excursions in 3.4 Seconds.

Time.	0.01 gm. pyrocatechol + 4 cc. 50 per cent tomato juice + 2 cc. H₂O.	0.01 gm. pyrocatechol + 4 cc. 50 per cent tomato juice + 2 cc. H₂O.	4 cc. 50 per cent tomato juice + 2 cc. H₂O.
11.15	0	0	0
11.30	0.10	0.10	0.55
11.45	0.20	0.20	0.60
12.00	0.35	0.30	0.90
12.15	0.20	0.20	0.75
12.30	0.15	0.20	0.75

* These results were copied from data secured in the course of a co-operative work with Professor R. W. Thatcher, of the University of Minnesota, and are published here with his permission.

The example above shows that we would not be justified in correcting our results obtained for the oxidation of pyrocatechol in the presence of tomato juice by mere subtraction of the figures obtained from the absorption of oxygen by the corresponding amount of tomato juice alone. The distribution of the chromogens is being studied in the laboratory at present and later a general paper will appear discussing their occurrence in plants and their significance.

We may conclude, therefore, that the figures which were secured throughout this work are the result of a number of factors, some having an augmenting effect on the end-result, others a depressing effect. There are undoubtedly other factors which come into play. The presence of reductases[6] and the concentration of hydrogen ions[7] undoubtedly have an influence on the result, but the nature of their action is too obscure as yet to allow any definite statements concerning them. While the question of reductases is far from being elucidated on account of lack of proper methods, the influence of the reaction of the medium is susceptible to accurate experimentation. The writer is engaged with the latter at present.

Although we have to recognize the retarding influence of the various factors mentioned, it is also evident that none of them are responsible for the prompt standstill invariably shown by the oxygen absorption. This phenomenon is peculiar to the agencies responsible for the oxygen absorption. If chemical compounds, the so called oxidases, are responsible for the absorption, then the oxidases become used up in the course of the reaction; if the peculiar condition of the colloids present accounts for the reaction, then a rearrangement into an inactive condition takes place during the reaction.

[6] Reed, G. B., Evidence for the General Distribution of Oxidases in Plants, *Bot. Gaz.*, 1915, lix, 407.

[7] Rose, D. H., Oxidation in Healthy and Diseased Apple Bark, *Bot. Gaz.*, 1915, lx, 55. (Also : dicated by some preliminary experiments of the writer.)

THE RELATIVE OXIDASE ACTIVITY OF DIFFERENT ORGANS OF THE SAME PLANT.[1]

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At the meeting of the American Society of Biological Chemists in 1913. a paper[2] was presented by the writer on the correlation of biological oxidizability and chemical constitution. By biological oxidizability is meant the ease with which certain aromatic compounds are oxidized by means of atmospheric oxygen in the presence of oxidase-active plant material.

The results given represent experiments carried out with the following eighteen reagents: pyrocatechol, resorcinol, hydroquinone, guaiacol, pyrogallol, phloroglucinol, α-naphthol, leuko-base of malachite green, aloin, benzidine, tyrosine, phlorhizin, o-cresol, m-cresol, p-cresol, o-toluidine, m-toluidine, and p-toluidine. Juices of shoots, tubers, and leaves of healthy potato plants and of plants having the curly-dwarf disease were the oxidase-active materials used. For convenience of comparison the results were presented graphically, the oxidase activities being laid off on the ordinates, and the organic compounds marked at equal intervals on the abscissæ. By connecting the points a line is obtained which gives a composite picture of the activity of the particular plant material. Six lines were obtained in this way, each representing the oxidizing power of a different type of potato material.

To quote from the paper referred to above[2]: "There is one striking feature about these six curves: They all show a similar trend. The elevations and depressions of each curve follow very closely those of the other curves. Let us take it for granted that the relationships found in the juice are indicative of conditions in the plant cell. Then we might say that the conditions in the cells which control the oxidizing powers toward these eighteen reagents are about the same in all parts of the potato plant. It may be that other plants show the same oxidase relationships or that each plant has its own type of curve representing its oxidase activities. The latter situation would be particularly interesting. We would gain access to some of the metabolic functions characteristic of the particular plant in question. Such specific enzyme relationships in animals have been dis-

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[1] Published by permission of the Secretary of Agriculture.

[2] Bunzell, H. H., *Jour. Biol. Chem.*, 1914, xvii, p. xxxvi.

covered in the case of the enzymes involved in nuclein metabolism (Walter Jones)."

Since the paper mentioned above was read, a number of other plants have been studied with respect to their oxidase activities. In this connection the potato, onion, tulip tree, spinach, and sugar beet have been studied. The experiments were carried out with the following organs: potato leaves, potato tubers, onion leaves, onion bulbs, tulip tree leaves, tulip tree buds, spinach leaves, and spinach roots. All results represent the mean of a number of experiments carried out on two or more samples.

The data for the potato are taken from a previous publication.[3] Only the figures representing the normal material were used. In the case of the onion (red danvers), the shoots and bulbs of the young plants (about 4 inches above ground) were dried in a desiccator over lime, powdered, and examined in the usual way. It turned out that neither bulbs nor foliage had any activity toward any of the reagents tried. The results obtained with the tulip tree material were taken from data obtained in cooperative work with Dr. William Crocker of the University of Chicago, at present Plant Physiologist in the Bureau of Plant Industry. The experiments were carried out with dried and powdered material, and the data were calculated on the basis of the original moisture content. In the case of the buds the data represent three collections made at different times of the year from five different trees, and two or three determinations with each reagent for each sample collected. Table I gives the average results.

TABLE I.

*Mean Results Obtained in the Study of the Oxidase Activity of Tulip Tree Buds. The Results Are Expressed in Terms of Units.**

Reagents.	Pyrogallol.	Pyrocatechol.	Hydroquinone.	Phlorhizin.	Guaiacol.	Tyrosine.	m-Cresol.	p-Cresol.	Eugenol.	Iso-eugenol.
Buds two pairs of scales off.	1.22	8.48	6.13	0.44	0.48	0.20	0.30	0.64	0.23	0.23
Inner pair of scales	1.15	6.70	3.04	0.40	0.39	0.17	0.29	1.04	0.18	0.21
Leaves	0.68	2.23	1.59	0.58	0.48	0.29	0.39	1.58	0	0

* "A unit is an oxidase solution of such strength that 1 liter of it will be capable of bringing about the consumption by pyrogallol of the equivalent of 1 gm. of hydrogen; *i.e.*, the consumption of 8 gm. of oxygen." (Bunzell, The Measurement of the Oxidase Content of Plant Juices, *U. S. Dept. of Agriculture, Bureau of Plant Industry, Bull. 238*, 1912, 40.)

[3] Bunzell, *Jour. Agric. Research*, 1914, ii, 373.

The results with spinach represent the mean of duplicates and triplicates carried out in three collections, roots and leaves (freed from midribs) separately (Table II).

TABLE II.

Results Obtained in the Study of Oxidase Activities of Spinach Plants. The. Results Are Expressed in Terms of Units.

Reagents.	Pyrogallol.	Pyrocate-chol.	Hydro-quinone.	Phlorhizin.	Guaiacol.	Tyrosine.	*m*-Cresol.	*p*-Cresol.	Eugenol.	Iso-eugen-ol.
Leaves...	0.080	0.242	0.023	0	0	0	0	0.084	0	0
Roots ...	0.166	0.312	0.590	0.274	0.197	0.562	0.300	2.434	0.003	0

In the case of sugar beets (young greenhouse plants) the data are less complete; reliable data for only six reagents are at hand (Table III). They are the following· pyrogallol, pyrocatechol, hydroquinone, phlorhizin, guaiacol, and tyrosine.

TABLE III.

Results Obtained in the Study of Oxidase Activities of Sugar Beets. The Results Are Expressed in Terms of Units.

Reagents.	Pyrogallol.	Pyrocate-chol.	Hydro-quinone.	Phlorhizin.	Guaiacol.	Tyrosine.
Roots..........	0.212	0.330	0.181	0.062	0.259	0.448
Leaves.........	0.196	0.273	0.196	0.080	0.234	0.354

In correlating the various data, it seemed interesting to examine the results relating to the same plant from the following points of view: (1) How does the relative oxidase activity exhibited by any plant material toward the reagents tried compare with the relative oxidase activity exhibited by other portions of the same plant toward the same reagents? (2) How does the general oxidase activity of certain plant organs compare with plant organs with fundamentally different functions?

To facilitate comparison of the data, it was necessary to record them in some graphic form. The circle was therefore divided into ten equal sections. Each diameter was made to represent a reagent and the activities of the plant materials toward the various reagents were laid off on the corresponding diameters.

By connecting the points obtained in this way a graph was se-
cured which represents the oxidase activity of the plant material
studied. By recording separately the activities of different parts
of the same plant on the same diagram, it is possible to make
immediate comparison. Diagrams are presented for the five
plants studied.
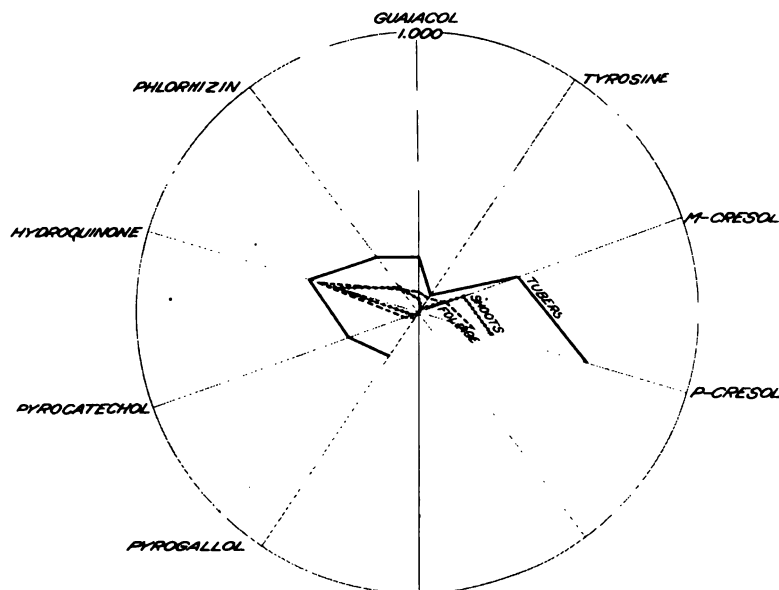
Fig. 1. Oxidase activities of different parts of the potato plant.

As can readily be seen there is a marked parallelism between
the graphs representing the oxidase activities of different parts of
the potato, tulip tree, and sugar beet. In the case of the onion
there is no activity shown by either leaves or roots. With spinach
the two graphs are entirely at variance. This non-parallelism
between the oxidase activities of spinach roots and spinach leaves,
however, may be only apparent. The activity of the leaves is
very slight and may become obscured by the presence of chromo-
gens, as pointed out in the previous paper.[4]

The diagrams show that the oxidase situation in the case of
spinach is entirely different from the oxidase situation in the case

[4] Bunzell, *Jour. Biol. Chem.*, 1916, xxiv, 91.
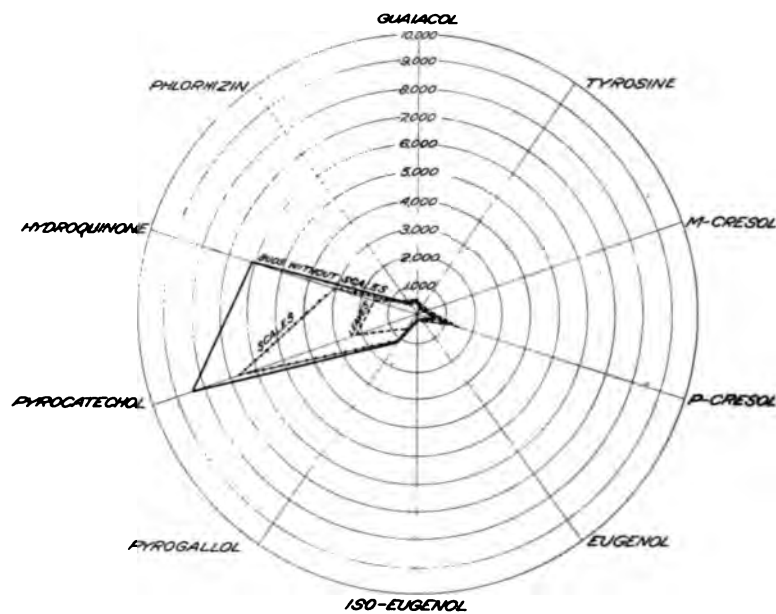
FIG. 2. Oxidase activities of different parts of the tulip tree.
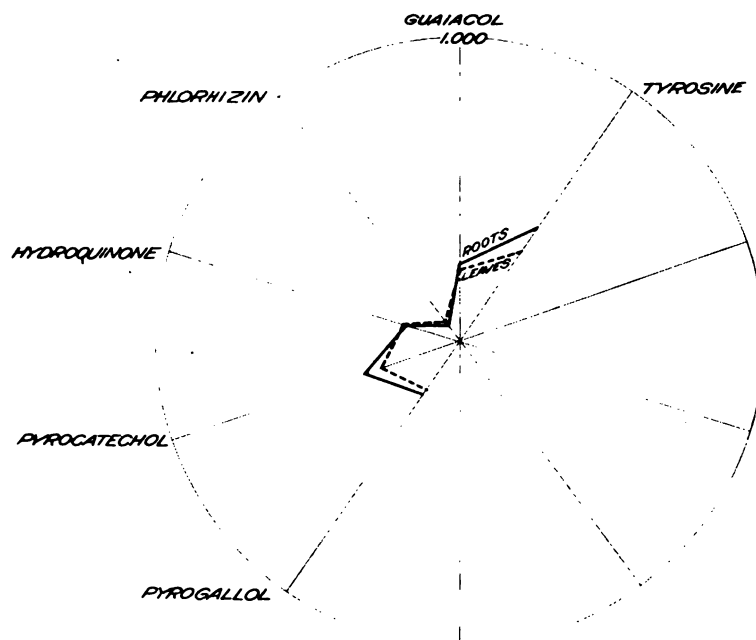
FIG. 3. Oxidase activities of different parts of the sugar beet plant.
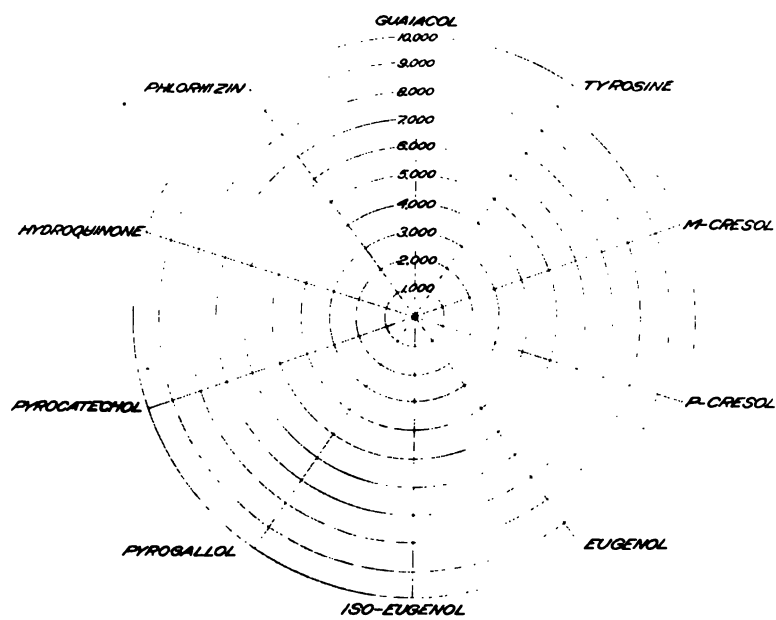
FIG. 4. Oxidase activities of different parts of the onion plant.
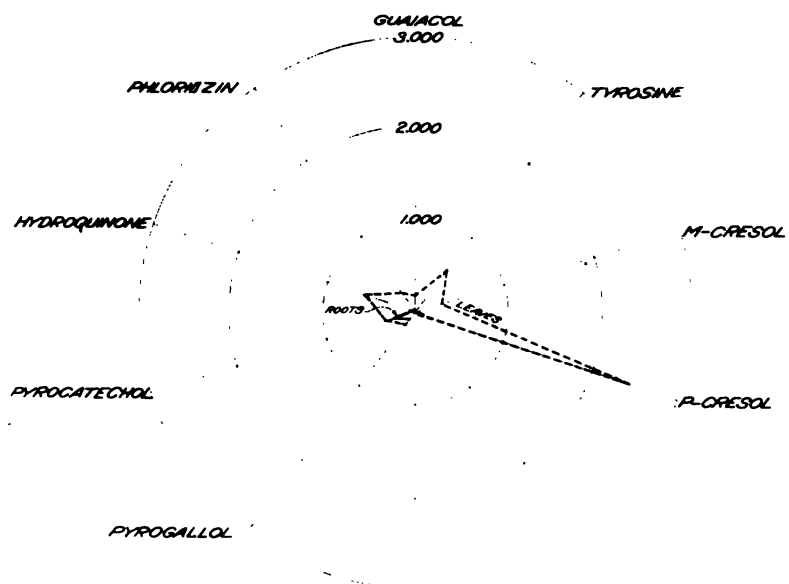
FIG. 5. Oxidase activities of different parts of the spinach plant.

of sugar beet, which in turn is different from that in the case of potato, tulip tree, or onion. The relative oxidase activity as manifested toward the reagents tried is approximately the same for the different parts of the same plant, but different for the different plants tried. It appears then that the second of the two assumptions made above, *viz.*, that each plant has its characteristic relationships, is correct.

What does this mean? To answer this question it would be necessary to know and to take account of all the factors entering into the complex of reactions summarized as oxidase activity. A number of these factors have been pointed out in the previous paper.[4] The fact that in general there is a well marked parallelism existing between the graphs of different organs of the same plant, would indicate that one of these factors must predominate strongly above the others. Our knowledge at present allows us only to surmise what this factor may be. If we assume that the oxidase activity of the plant juices is due to the presence of specific oxidases, as Bach and Chodat claim, then it will be difficult to understand why the oxidation of each reagent should be equally lower in some parts of a plant as compared with other parts of the same plant. If, on the other hand, we assume that the oxidations in the presence of the plant material are carried out principally by virtue of the physicochemical conditions created by the addition of the plant material, an explanation seems nearer at hand. In fact, Traube[5] in his paper on catalysis recognized surface tension as one of the important factors in the acceleration of biochemical reactions. The oxidase activities observed might simply be due to an increased concentration of the oxidizable material or of oxygen, or of both in the layers adjacent to certain colloidal particles. A similar view has been expressed by H. E. Armstrong and coworkers.[6] The particular nature of the colloidal particles present might be a determining factor for the relative concentration of the various oxidizable substances, and thereby for the relative extent of oxidation of these reagents. The actual concentration of the colloids, on the other hand, might be responsible for the general intensity of the oxidase activity. Taking it for

[5] Traube, J., *Arch. f. d. ges. Physiol.*, 1913, cliii, 309.

[6] Armstrong, H. E., Benjamin, M. S., and Horton, E., *Proc. Roy. Soc.*, *Series B*, 1912-13, lxxxvi, 328.

granted then that a certain group of colloids of this nature is being developed by the cells of a certain plant, it would be easy to see why cells in certain organs, generally more active, should produce more of such colloids than others generally less active.

The capacity of certain organs to manufacture colloids of a highly specific nature is illustrated by the work of Reichert.[7] He carried out extensive investigations on starches of different genera, species, varieties, and hybrids, as well as on starches obtained from different parts of the same plant. He studied the appearance of the starch grain, the color reactions, and certain physical constants of the starches, and concluded that every genus and species has a form of starch which is specific and constant in relation to that genus or species. It is probable that similar relationships will be found in the characteristics discussed in this paper. If the oxidase activities observed are due to the presence of certain colloids, this situation is probable.

There remains another aspect of the situation to be discussed. In the metabolism of the plant there are innumerable compounds which are being oxidized in the cells, presumably with the aid of oxidase action. Some of these compounds are probably related to the various aromatic compounds mentioned in this paper. In the light of the findings stated in this paper it would seem that the capacity of the plant to oxidize these various compounds would be characteristic of the particular plant, but would vary in intensity, and in intensity only, in the different parts of the same plant. This line of reasoning would furnish us with direct proof of the existence of a distinct and characteristic type of metabolism for each plant. It remains to be seen to what extent plants of the same genus or species resemble each other in their metabolism.

[7] Reichert, E. T., The Differentiation and Specificity of Starches in Relation to Genera, Species, etc., *Carnegie Institution of Washington, Publication No. 173*, 1913. Also *Science*, 1914, xl, 649.

CEPHALIN.[1]

III. CEPHALIN OF THE EGG YOLK, KIDNEY, AND LIVER.

PRELIMINARY PAPER.

By P. A. LEVENE and C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The principal object of the present investigation was the search for a convenient source of cephalin. It is true that the brain tissue contains a high proportion of this substance, but it also contains a variety of other phosphatides. Experience has shown that because of this the purification of cephalin from the brain tissue met with great obstacles. It was hoped that in some other organ the distribution of phosphatides might be so much in favor of cephalin that it could be readily freed from impurities. Unfortunately this expectation was not realized for the present. The brain still remains the most convenient source of cephalin. On the other hand, the results of the present work have shown that the cephalins obtained from various organs and from egg yolk possess identical properties and composition, and hence the results of the structural analysis of one will apply to all.

The following table contains the results of the ultimate analysis of cephalin prepared by different workers.

Author.	Source.	C	H	N	P
Levene and West[1]	Brain	60.49	9.52	1.96	3.80
Stern and Thierfelder[2]	Egg yolk	59.68	9.74	1.57	3.64
Levene and West	"	60.00	9.62	1.78	3 69
Frank[3]	Liver	57.10	9.62	1.72	3.91
Levene and West	"	60.33	9.01	1.86	3.75
" " "	Kidney	60.17	8.95	1.70	3.65

[1] Levene, P. A., and West, C. J., *Jour. Biol. Chem.*, 1913-14, xvi, 419; 1916, xxiv, 41.

[2] Stern, M., and Thierfelder, H., *Z. f. physiol. Chem.*, 1907, liii, 379.

[3] Frank, A., *Biochem. Z.*, 1913, 1, 277.

From the table it is seen that from egg yolk the material obtained by us did not differ from that prepared by Stern and Thierfelder, although we employed a more rigid procedure of purification.   On the other hand, the substance isolated by us from the liver was undoubtedly in a higher state of purity than that described by Frank, and finally in regard to the kidney the present is the first report of the isolation of cephalin from that organ.

Work on other organs is in progress.

EXPERIMENTAL PART.

Egg Yolk Cephalin.

Earlier workers and many of the later workers in lipoid chemistry considered all of the lipoid fraction of egg yolk as lecithin.[4] Erlandsen[5] was the first to question the composition of the lecithin fraction.   During the course of his work on the lipoids of heart muscle, he attempted to isolate cuorin from egg yolk, but was unsuccessful.   In the same year Stern and Thierfelder[2] reported the isolation of an alcohol-insoluble fraction from the so called lecithin.   The compound obtained from fresh eggs resembled cephalin in its properties, and analyzed for the theoretical cephalin composed of stearic and cephalinic acids, glycerophosphoric acid, and amino-ethyl alcohol.[6]   The cephalin from a commercial egg preparation, however, showed the composition found by other authors; namely, C, 59.68; H, 9.74; N, 1.57; P, 3.64.   The only explanation offered for the different results was that possibly the age of the egg had some effect.   We have repeated the work on the egg yolk, using commercial egg yolk powder.

Alcohol Extraction.

Egg yolk powder was extracted four or five times with hot alcohol, the alcoholic extract concentrated and cooled at 0°.

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[4] For example, Serono, C., and Palozzi, A. (*Arch. farm. sperim.*, 1911, xi, 553), state that 11–12 per cent of the egg yolk is lecithin, but mention no other lipoid.   It is interesting in this connection to compare the work of Barbieri, N. A. (*Compt. rend. Acad. d. sc.*, 1910, cli, 405), who denies the existence of free or bound lecithin in the egg yolk.

[5] Erlandsen, A., *Z. f. physiol. Chem.*, 1907, li, 150.

[6] See Levene and West, *Jour. Biol. Chem.*, 1916, xxiv, 41.

The egg oil and lipoids separated as a semisolid mass. Since it was impossible to filter this, even at 0°, the entire product was stirred with a large quantity of dry acetone. This dissolved the egg oil and considerable quantities of lipoid. The acetone extract was concentrated and again extracted with dry acetone, and the process repeated until the oil dissolved completely in the acetone. All the lipoid fractions were then extracted with ether, and the small amount of sphingomyelin was removed by filtration or centrifugation. The ether-soluble fraction was then separated by precipitation with absolute alcohol into the cephalin (alcohol-insoluble) and lecithin (alcohol-soluble) fractions.

This crude cephalin fraction contained 1.8 per cent nitrogen. When treated with alcohol at 60–70°, as recommended by Mac-Lean for the preparation of cuorin, a product was obtained with the same composition:

0.500 gm. substance neutralized 6.18 cc. 0.1 N acid.
0.300 "   "   gave 0.0390 gm. $Mg_2P_2O_7$.

	N	P
Found	1.72	3.62

The material was then precipitated from alcohol at 60°, in the same manner as described for the purification of brain cephalin. The two fractions (I, insoluble, and II, soluble) showed the same composition:

I. 0.500 gm. substance neutralized 6.34 cc. 0.1 N acid.
   0.300 "   "   gave 0.0386 gm. $Mg_2P_2O_7$.
II. 0.500 "   "   neutralized 6.48 cc. 0.1 N acid.
    0.300 "   "   gave 0.0404 gm. $Mg_2P_2O_7$.

	Found:	
	N	P
I	1.75	3.60
II	1.81	3.74

Finally, the ether solution was shaken with dilute hydrochloric acid and the resulting solution repeatedly precipitated with acetone.

0.500 gm. substance neutralized 6.33 cc. 0.1 N acid.
0.300 "   "   gave 0.0398 gm. $Mg_2P_2O_7$.
0.1484 "   "   " 0.3265 " CO_2 and 0.1276 gm. H_2O.
0.300 "   "   " 0.0662 " AgI (glycerol determination).

	C	H	N	P	Glycerol.
Found	60.00	9.62	1.78	3.69	8.65

Ether Extraction.

In order to determine whether the initial solvent used had any effect upon the composition of the alcohol-insoluble fraction, a second lot of egg powder was extracted repeatedly with ether at room temperature. The combined ether extract was treated as given above. The fraction, insoluble in alcohol at 60°, had the following composition:

0.500 gm. substance neutralized 6.53 cc. 0.1 N acid.
0.300 "   "   gave 0.0400 gm. $Mg_2P_2O_7$.
0.1516 "   "   "   0.3327 " CO_2 and 0.1273 gm. H_2O.
0.300 "   "   "   0.0660 " AgI (glycerol determination).

	C	H	N	P	Glycerol.
Found	59.86	9.40	1.82	3.70	8.65

Commercial Lecithin.

The same cephalin has also been obtained from a sample of commercial lecithin. This was found to consist of considerable egg oil, together with sphingomyelin, cephalin, and lecithin. The cephalin fraction, insoluble in alcohol at 60°, had the same composition as the two samples analyzed above, and figures need not be given.

Lead Salt.

The lead salt was prepared by the method given in our second paper and had the following composition:

0.500 gm. substance neutralized 3.01 cc 0.1 N acid.
0.300 "   "   gave 0.0262 gm. $Mg_2P_2O_7$.

	N	P
Found	1.05	2.43

Kidney Cephalin.

A review of the work on the lipoids of the kidney has been given by Forbes and Keith.[7] The most important and complete investigation is that of MacLean.[8] In the present work cephalin

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[7] Forbes, E. B., and Keith, M. H., Phosphorus Compounds in Animal Metabolism, Wooster, 1914, 141.

[8] MacLean, H., *Biochem. Jour.*, 1912, vi, 333.

was obtained from the kidney by the same method as that used in the work on brain cephalin. The product insoluble in alcohol at 60° had the following composition:

0.500 gm. substance neutralized 6.08 cc. 0.1 N acid.
0.300 "   "   gave 0.0394 gm. $Mg_2P_2O_7$.
0.1050 "   "   "   0.2316 " CO_2 and 0.0840 gm. H_2O.

	C	H	N	P
Found	60.17	8.95	1.70	3.65

The same results were obtained when the dried kidney material was extracted with ether. Because of the difficulty of purifying cephalin in general, no other details of the work will be given here.

Liver Cephalin.

The first alcohol-insoluble lipoid isolated from the liver was called jecorin.[9] The widely divergent results obtained by various observers indicated clearly that this substance was a mixture.[10] Baskoff[11] obtained a cuorin-like substance which he called *heparphosphatide;* this had a N : P ratio of about 1 : 1.5. Still later Frank[10] obtained a compound which resembled cephalin in its composition and properties.

The liver was worked up in the same general way as the other organs. The ether-soluble material of the alcoholic extract was precipitated repeatedly with alcohol and then with acetone. The cephalin was then emulsified with water, precipitated with 10 per cent hydrochloric acid, the precipitate taken up in ether and repeatedly precipitated with dry acetone. Solutions in amyl alcohol or ethyl acetate deposited the cephalin upon cooling, without

[9] Drechsel, E., *Jour. f. prakt. Chem.,* 1886, xxxiii, 425. Baldi, D., *Arch. Physiol.,* Suppl., 1887, 100. Jacobsen, A., *Skand. Arch. f. Physiol.,* 1895, vi, 262. Manasse, P., *Z. f. physiol. Chem.,* 1895, xx, 478. Drechsel, *Z. f. Biol.,* 1896, xxxiii, 86. Henriques, V., *Z. f. physiol. Chem.,* 1897, xxiii, 244. Meinertz, J., *Z. f. physiol. Chem.,* 1905, xliv, 371. Siegfried, M., and Mark, H., *Z. f. physiol. Chem.,* 1905, xlvi, 492. Waldvogel and Tintemann, *Z. f. physiol. Chem.,* 1906, xlvii, 129. Mayer, P., *Biochem. Z.,* 1906, i, 81. Baskoff, A., *Z. f. physiol. Chem.,* 1908, lvii, 395; 1909, lxi, 426; 1909, lxii, 162.

[10] For table of analyses and discussion, see Frank, A., *Biochem. Z.,* 1913, l, 277.

[11] Baskoff, *Z. f. physiol. Chem.,* 1908, lvii, 395.

any purification (see Baskoff). The material had the following composition:

0.500 gm. substance neutralized 6.8 cc. 0.1 N acid.
0.300 "   "   gave 0.0404 gm. $Mg_2P_2O_7$.
0.1622 "   "   "   0.3789 " CO_2 and 0.1306 gm. H_2O.

	C	H	N	P
Found	60.33	9.01	1.86	3.75

This material thus has the composition of cephalin. However, when fractionation out of alcohol at 60° was tried, it was found that this material could be separated into two rather constant fractions. The alcohol-soluble fraction had the composition of cephalin, while the alcohol-insoluble fraction contained a higher nitrogen content. In one case we were able to obtain a N : P ratio of nearly 2 : 1. We are not prepared, as yet, to discuss the significance of this fraction.

The so called cephalin, obtained above, was dissolved in ether and poured, with stirring, into alcohol heated to 60°. The filtrate was concentrated, and the material again precipitated in the same way. The third precipitation gives a body with the same N : P ratio, both in the precipitate and in the filtrate. This substance has all the properties of cephalin.

0.500 gm. substance neutralized 5.9 cc. 0.1 N acid.
0.300 "   "   gave 0.0394 gm. $Mg_2P_2O_7$.
0.1532 "   "   "   0.3404 " CO_2 and 0.1260 gm. H_2O.

	C	H	N	P
Found	60.6	9.20	1.65	3.66

Lead Salt.

The lead salt was prepared as usual. The first analysis is of a sample which had been washed with acetone; the second, of a sample which had been extracted with boiling methyl alcohol.

I. 0.500 gm. substance neutralized 3.96 cc. 0.1 N acid.
    0.300 "   "   gave 0.0250 gm. $Mg_2P_2O_7$.
II. 0.500 "   "   neutralized 3.77 cc. 0.1 N acid.
    0.300 "   "   gave 0.0282 gm. $Mg_2P_2O_7$.

		Found:
	N	P
I	1.10	2.32
II	1.06	2.61

THE DETERMINATION OF UREA BY THE UREASE METHOD.

By DONALD D. VAN SLYKE and GLENN E. CULLEN.

(From the Hospital of The Rockefeller Institute for Medical Research.)

In a recent number of this *Journal* Fiske[1] recommends as an improvement to the accuracy of our technique for urea determination[2] the use of a slower rate of aeration in driving the ammonia over into the standard acid, and the consequent prolongation of the time for aeration to an hour, in order to insure complete absorption of the ammonia by the acid. This modification and the consequent loss of time, however, are unnecessary if the directions given in our paper are followed; the accuracy attainable is limited only by that of the measurements and standard solutions. The conditions necessary for both complete and rapid absorption were studied rather exhaustively in experiments which it did not seem, at the time, essential to publish, but it now appears that certain of the principles on which our procedure was based must be stated more clearly.

1. One of the important factors in complete absorption is the height of the absorbing column of standard acid. We utilized as containers of the absorbing solution test-tubes of 100 cc. capacity and the usual shape (about 25 x 200 mm.). In such tubes 25 cc. of acid make a column 50 to 60 mm. high. Instead of these Fiske used "bottles," in which the column of acid is presumably much shorter. This change is the most obvious reason for the longer period of aeration required.

[1] Fiske, C. H., *Jour. Biol. Chem.*, 1915, xxiii, 455.
[2] Van Slyke, D. D., and Cullen, G. E., A Permanent Preparation of Urease, and Its Use in the Determination of Urea, *Jour. Biol. Chem.*, 1914, xix, 211.

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2. Another point which may bear expansion is the reason for
our statement (page 221): "If one uses *a moderate current of air
for the first minute* every particle of ammonia is absorbed."

The necessity for caution at the start is that, with a given air
current, the rate at which ammonia is carried over at the begin-
ning of the aeration is enormously faster than afterwards. Con-
sequently what caution is required should be concentrated on
retarding the first burst of ammonia evolution. After that is
over, it is almost impossible, with the apparatus and conditions
described in our paper, to drive the ammonia over into the acid so
fast that any of it escapes absorption. It does no harm, of
course, to prolong the period of relatively slow aeration, but it is
unnecessary. It is essential only that the fact be kept in mind
that the first stage of aeration is the one that requires all the
caution in regulating the air current. After this period is past,
it is only a waste of time not to use as fast a current as is possible
without loss of liquid from spattering. A much faster current
can be used with a drop of caprylic alcohol to prevent foaming
than with any other practicable agent, such as kerosene, for this
purpose with which we are acquainted. We usually employ an
air current of about 5 liters per minute, which insures complete
aeration of the ammonia in 15 minutes.

Experiment 1.—The relatively high rate at which ammonia is evolved
at the beginning of the aeration, and the constantly decreasing rate during
the later stages, are indicated by the following experiment. 5 cc. portions
of 0.0991 N ammonium sulfate (5 cc. = 24.78 cc. 0.02 N ammonia) were
measured into each of a series of tubes arranged for aerating as illustrated
on page 217 of our former paper, with the addition that a second absorption
tube, containing 1 cc. of 0.01 N acid plus 10 cc. of water, was inserted after
each pair of tubes. 5 cc. portions of 1:1 potassium carbonate solution
were added, and the aeration was conducted with an air current of 5.3
liters per minute from the start. The ammonia was absorbed into 30 cc.
portions of 0.02 N acid. At different intervals the successive sets of ab-
sorption tubes were disconnected and the ammonia which had been driven
over was titrated. The results are given in Table I.

The air current was determined by Kober's[3] scheme of measuring the
time required to draw 10 liters of water from one bottle into another. The
water levels in both were so arranged that positive water pressure during

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[3] Kober, P. A., and Graves, S. S., *Jour. Am. Chem. Soc.*, 1913, xxxv,
1594.

the first half of the transfer was balanced by negative during the second; and the connecting tube was so large (1.5 cm. inner diameter) that the flow of water was not appreciably retarded.

The analyst will find it worth while to standardize in this manner the air current and calculate the length of time required to drive 100 liters of air through the apparatus; as this amount with the apparatus described in our paper appears sufficient, with a good margin of safety, to drive over all the ammonia.

TABLE I.

Time of aeration.	Volume of air passed.	0.02 n HCl neutralised in first receiving tube.	0.02 n HCl neutralised in second receiving tube.	Total HCl neutralised.
min.	*liters*	*cc.*	*cc.*	*cc.*
1	5.3	13.5	0.08	13.58
2	10.6	20.3	0.08	20.38
3	15.9	22.8	0.08	22.88
5	26.5	24.1	0.08	24.18
10	53.0	24.6	0.09	24.69
15	79.5	24.7	0.09	24.79
25	132.5	24.7	0.08	24.78

At the beginning of the aeration the speed of evolution was so great that more than 55 per cent of the total ammonia was driven over in the first minute, 0.32 per cent being driven past the first receiving tube and caught in the second. During the second minute 27 per cent of the total ammonia was driven over. None passed the first tube during the second or following minutes. It is evident, therefore, that if the rate of evolution during the first 2 minutes had been reduced by cutting down the air current to one-half, no ammonia at all would have been lost. As it was, with a full air current from the start, the loss was only 0.3 per cent of the total, 99.7 per cent being retained in the first receiving tube. The amount of ammonia aerated was equal to the maximum ordinarily obtained in urine analyses.

It is a point of interest that, air current and other conditions being constant, the rate at which ammonia is driven over is exactly proportional to the amount present. The rate of evolution follows the same law $\frac{dx}{dt} = k\ (a - x)$ as the rate of a mono-

molecular reaction. Thus, taking a (total amount of ammonia present) equivalent to 24.78 cc. of 0.1 N acid, x being the amount driven over after t minutes, we have:

TABLE II.

t	x	$k = \frac{1}{t} \log_e \frac{a}{a-x}$	$k\,(a-x)$ Calculated rate of aeration after t minutes.
	Cc. 0.02 N NH₃		Cc. 0.02 N NH₃ per min.
min.			
0			20.2
1	13.58	0.794	9.0
2	20.38	0.867	3.6
3	22.88	0.857	1.5
5	24.18	0.744	0.5

Average.......................... 0.82

The value, 0.82, of k indicates that at any given moment ammonia was being removed at the rate per minute of 82 per cent of the ammonia present.

The figures in the last column are calculated from the velocity equation $\frac{dx}{dt}$ = rate of aeration = $k\,(a - x)$ = 0.82 (24.78 $-$ x). They show that after 1 minute the rate of aeration had fallen parallel with the still remaining amount of ammonia, $a - x$, to less than half the initial rate, and that after 2 minutes the rate had fallen to one-sixth the initial velocity.

Experiment 2.—In order to ascertain the effect of using a half-speed air current during the first minute, four sets of tubes were aerated for 15 minutes in precisely the same manner as in the above experiment, except that during the first minute an air stream of 2.6 liters per minute was used, the 5.3 liter current being applied after 1 minute's aeration. The amounts of 0.01 N acid neutralized in the guard tubes were 0.04 to 0.05 cc., equivalent to 0.02 to 0.025 cc. of 0.02 N acid, or 0.08 to 0.10 per cent of the total ammonia, 99.90 to 99.92 per cent being absorbed by the 0.02 N acid in the first receiving tubes.

As an error not exceeding 1 part per 1,000 may be regarded as negligible for most purposes, we believe that no alteration need be made in the directions in our first paper for the technique of

aeration. If the analyst wishes, however, to make sure that even less than 1 part of ammonia per 1,000 escapes absorption, he may continue the half-current for 2 minutes instead of 1.

3. Since our former publication we have found, like Fiske, that addition of potassium carbonate in approximately saturated solutions, to set free ammonia for aeration, is preferable to its addition in solid form, and have privately recommended this modification. The difference is one of convenience rather than accuracy. Contrary to Fiske's assumption, the increase in volume of liquid from 5 cc. to 10 cc. does not significantly retard the evolution of ammonia during aeration. As we stated (page 221) after systematic tests, "The volume of the solution (in a 100 cc. test-tube) from which ammonia is being removed can be varied from 5 cc. to 25 cc. without appreciably altering the time required to drive off the ammonia," as long as the solution contains potassium carbonate in a concentration of at least 1 gm. to 2 cc. of solution.

4. Although, as we showed and Fiske repeats, urease in water extracts containing primary and secondary phosphate preserves most of its activity for several weeks when kept at 0°, ammonia is likely to form in such extracts, even at 0°, and in sufficient amounts to cause relatively large errors, particularly in blood analyses. The loss of activity also is rather insidious, for the reason that an extract which has remained for a long time almost without change loses activity at an unexplainably rapid rate after it has once begun to deteriorate. The safest way is to use a standardized preparation of dry urease, and prepare fresh solutions daily as required.

5. We have found it necessary, especially in blood analyses, to correct for ammonia in the reagents. The chief source of the correction is the potassium carbonate. Blank determinations are run on every lot of reagents, using 1 cc. of 10 per cent urease solution, 5 cc. of the 0.6 per cent phosphate solution, and 10 cc. of saturated potassium carbonate solution, and aerating into 0.01 N acid. The correction obtained has been as high as 0.12 cc. of 0.01 N acid, but with the carbonate now in use (Merck's U. S. P. viii) is 0.05 cc. of 0.01 N acid.

6. In closing we mention one essential precaution which we have always observed as a matter of course, but neglected to state in our original paper. As a result at least one colleague

molecular reaction. Thus, taking a (total amount of ammonia present) equivalent to 24.78 cc. of 0.1 N acid, x being the amount driven over after t minutes, we have:

TABLE II.

t	x	$k = \frac{1}{t} \log_e \frac{a}{a-x}$	$k\,(a-x)$ Calculated rate of aeration after t minutes.
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Average.......................... 0.82

The value, 0.82, of k indicates that at any given moment ammonia was being removed at the rate per minute of 82 per cent of the ammonia present.

The figures in the last column are calculated from the velocity equation $\frac{dx}{dt}$ = rate of aeration = $k\,(a-x)$ = 0.82 (24.78 − x). They show that after 1 minute the rate of aeration had fallen parallel with the still remaining amount of ammonia, $a - x$, to less than half the initial rate, and that after 2 minutes the rate had fallen to one-sixth the initial velocity.

Experiment 2.—In order to ascertain the effect of using a half-speed air current during the first minute, four sets of tubes were aerated for 15 minutes in precisely the same manner as in the above experiment, except that during the first minute an air stream of 2.6 liters per minute was used, the 5.3 liter current being applied after 1 minute's aeration. The amounts of 0.01 N acid neutralized in the guard tubes were 0.04 to 0.05 cc., equivalent to 0.02 to 0.025 cc. of 0.02 N acid, or 0.08 to 0.10 per cent of the total ammonia, 99.90 to 99.92 per cent being absorbed by the 0.02 N acid in the first receiving tubes.

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aeration.   If the analyst wishes, however, to make sure that even less than 1 part of ammonia per 1,000 escapes absorption, he may continue the half-current for 2 minutes instead of 1.

3. Since our former publication we have found, like Fiske, that addition of potassium carbonate in approximately saturated solutions, to set free ammonia for aeration, is preferable to its addition in solid form, and have privately recommended this modification. The difference is one of convenience rather than accuracy.   Contrary to Fiske's assumption, the increase in volume of liquid from 5 cc. to 10 cc. does not significantly retard the evolution of ammonia during aeration.   As we stated (page 221) after systematic tests, "The volume of the solution (in a 100 cc. test-tube) from which ammonia is being removed can be varied from 5 cc. to 25 cc. without appreciably altering the time required to drive off the ammonia," as long as the solution contains potassium carbonate in a concentration of at least 1 gm. to 2 cc. of solution.

4. Although, as we showed and Fiske repeats, urease in water extracts containing primary and secondary phosphate preserves most of its activity for several weeks when kept at 0°, ammonia is likely to form in such extracts, even at 0°, and in sufficient amounts to cause relatively large errors, particularly in blood analyses.   The loss of activity also is rather insidious, for the reason that an extract which has remained for a long time almost without change loses activity at an unexplainably rapid rate after it has once begun to deteriorate.   The safest way is to use a standardized preparation of dry urease, and prepare fresh solutions daily as required.

5. We have found it necessary, especially in blood analyses, to correct for ammonia in the reagents.   The chief source of the correction is the potassium carbonate.   Blank determinations are run on every lot of reagents, using 1 cc. of 10 per cent urease solution, 5 cc. of the 0.6 per cent phosphate solution, and 10 cc. of saturated potassium carbonate solution, and aerating into 0.01 N acid.   The correction obtained has been as high as 0.12 cc. of 0.01 N acid, but with the carbonate now in use (Merck's U. S. P. viii) is 0.05 cc. of 0.01 N acid.

6. In closing we mention one essential precaution which we have always observed as a matter of course, but neglected to state in our original paper.   As a result at least one colleague

has had much unnecessary trouble. If a test-tube and stopper are used as container, first for the strong carbonate solution, then, during a subsequent aeration, for the 0.02 N or 0.01 N acid solution, the tube or stopper, no matter how thoroughly washed with distilled water, is likely to carry a trace of alkali into the highly dilute acid and appreciably affect results. Therefore separate sets of tubes and stoppers must be employed as containers for the standard acid, and these must not be used for alkaline solutions. In case a tube that has been used for an alkaline solution is employed for standard acid, both tube and stopper are washed first with dilute acid, then with water.

It may be well also to call attention to the fact that the titration value of 0.01 N or 0.02 N alkali in ordinary glass vessels increases perceptibly from week to week, because the solution gradually dissolves more alkali from the glass. This action is particularly rapid in such a long narrow vessel as a burette. Alkali of 0.01 N concentration may appreciably increase its titration value as the result of standing for even a day in a burette.

STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL.

XIV. THE EFFECT OF FEEDING PITUITARY SUBSTANCE AND CORPUS LUTEUM SUBSTANCE ON EGG PRODUCTION AND GROWTH.

By RAYMOND PEARL.

(From the Biological Laboratory of the Maine Agricultural Experiment Station, Orono (Paper No. 91), and the Organotherapeutic Laboratory of Armour and Company, Chicago.)

I.

In the preceding paper in this series[1] it was shown that the injection of the substance or extract of the anterior lobe of the pituitary body into the peritoneal cavity of the domestic fowl failed entirely to activate the completely resting ovary. On general grounds, as set forth in the paper cited, there appeared to be many reasons for thinking it probable that pituitary substance might activate the ovary. On those grounds it was decided to extend the experiments with this substance somewhat farther, and along a different line. It is a well known fact, of course, that the specific physiological effects of certain endocrinal glands are produced when the gland substance is fed. Perhaps the most conspicuous example of this is found in the case of thyroid gland therapy. On the other hand, it is not to be forgotten that the products of endocrinal glands normally have a parenteral introduction into the body fluids. It is this consideration which has made intraperitoneal or intravenous injection the method of choice in the writer's experiments in the field hitherto.[2]

Because of the negative results with pituitary substance in the injection experiments it seemed worth while to try administration

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[1] Pearl, R., and Surface, F. M., *Jour. Biol. Chem.*, 1915, xxi, 95.

[2] Pearl and Surface, *Jour. Biol. Chem.*, 1914, xix, 263; 1915, xxi, 95; *Maine Agricultural Experiment Station, Report for 1915*, 65.

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per os. It is the purpose of this paper to record the results of certain experiments by this method.

The general plan of the first series of experiments to be tried was the following. With a group of hens sufficiently large to give reliable results, at a time in the laying year when under normal circumstances the rate of egg production is declining, will an administration of pituitary substance (anterior lobe) *per os* bring about an increase in the rate of fecundity? The experiment was carried out with three pens, each containing fifteen pure-bred barred Plymouth Rock hens, approximately 15 months of age at the beginning. In sorting the birds for the experiment into the three pens the attempt was made to get as even a distribution as possible, so that those in one pen should be substantially equal to those in the other pens in respect to innate laying ability, physiological condition at the time, body weight, breed, and general disposition.

To each bird in two of these pens (Pens A and B) was administered each day during the experiment (see below for dates) a No. 2 gelatin capsule filled with some mixture of pituitary body (anterior lobe) substance and milk sugar, or milk sugar alone. The data on the mixtures used and dates fed are as follows:

Pen A. Pituitary Pen.—July 10–24. Capsules filled with a mixture of three parts by weight of lactose and one part by weight of pituitary body (anterior lobe) substance. Average weight of filled capsule, 0.25 gm. This means, on the average, 0.0625 gm. desiccated anterior lobe substance per bird per day during this period.

July 25–Aug. 10. Capsules filled with a mixture of three parts lactose and two parts anterior lobe substance. This doubled the dose of the latter substance.

The pituitary substance used was the same as that used in the preceding experiments,[1] and prepared in the Organotherapeutic Laboratory of Armour and Company, especially for this work. Its preparation has been described in the paper cited and need not be repeated here.

Pen B. Lactose Control Pen.—July 10–Aug. 10. Capsules filled with milk sugar (lactose) only.

Pen C. Complete Control Pen.—July 10–Aug. 10. The birds in this pen received no capsules.

The results of the experiments ·are set forth in Table I and Fig. 1. All the birds in the experiment were trap-nested. These records are tabulated for (a) a preliminary period of 3 months and 9 days before the beginning of the pituitary feeding, (b) the experimental period, and (c) an after-period of 20 days. I have grouped the daily production into 5 day periods. The tabulated figures give the total number of eggs laid by each pen of birds in consecutive 5 day periods, the total production being supposed to be concentrated on the middle day of the period.

The data of Table I are shown graphically in Fig. 1.

TABLE I.

The Results of Feeding Pituitary Body (Anterior Lobe) Substance to Laying Hens.

Date.	Eggs laid in Pen A. (Pituitary.)	Eggs laid in Pen B. (Lactose control.)	Eggs laid in Pen C. (Complete control.)	Date.	Eggs laid in Pen A. (Pituitary.)	Eggs laid in Pen B. (Lactose control.)	Eggs laid in Pen C. (Complete control.)
Preliminary period.							
Apr. 3	49	44	52	July 2	49	43	39
" 8	47	45	55	" 7	54	40	37
" 13	45	46	46				
" 18	46	48	43	Experimental feeding began July 10.			
" 23	49	40	37	July 12	43	33	35
" 28	45	33	32	" 17	51	35	40
May 3	56	41	34	" 22	45	35	40
" 8	56	42	31	" 27	43	40	35
" 13	54	52	45	Aug. 1	42	37	36
" 18	51	48	53	" 6	34	45	39
" 23	42	51	43				
" 28	55	54	42	Experimental feeding ended Aug. 10.			
June 2	49	48	43	Aug. 11	40	36	34
" 7	51	42	44	" 16	39	38	46
" 12	42	37	40	" 21	43	28	39
" 17	36	41	40	" 26	36	32	40
" 22	39	42	41	Two birds in Pen A died during the experiment; one on Aug. 4, the other on Aug. 20.			
" 27	48	49	40				

From this table and diagram it is clear that:

1. The general trend of egg production in all the forty-five birds in the experiment in the period from Apr. 1 to Sept. 1 is downwards. This is the normal course of egg production at this season of the year.[3] That the general trend is downward in

[3] Compare Pearl and Surface, *U. S. Dept. of Agriculture, Bureau of Animal Industry, Bull. 110*, pt. ii, 1911, 81.
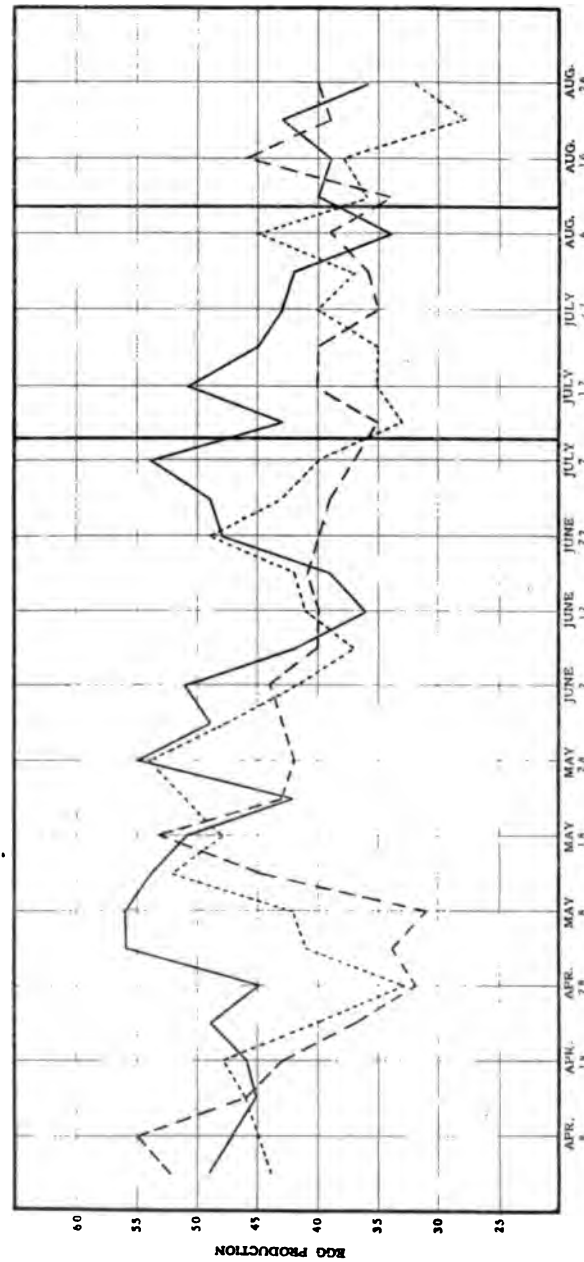
FIG. 1. Showing the egg production in experimental pens A, B, and C. Pen A, solid line; Pen B, dotted line; Pen C, broken line. The heavy vertical lines mark off the three periods of the experiment; *viz.*, the preliminary period, the feeding period, and the after-period.

these experimental flocks in the period covered by Table I is proved by the equations of the best fitting straight lines, fitted by the method of least squares, to these data. These equations are:

$$\text{Pen A} : y = 51.6184 - 0.3646\ x$$
$$\text{Pen B} : y = 47.6276 - 0.2400\ x$$
$$\text{Pen C} : y = 44.4207 - 0.2400\ x$$

where y denotes eggs produced, and x days after Apr. 1. The x term in all three equations is negative, demonstrating the general downward trend of the production, apart from the minor fluctuations.

2. The pituitary-fed birds fell off in production steadily during the period of experimental feeding (July 10–Aug. 10). For some weeks before, and at the beginning of the feeding period the Pen A birds had been laying at a somewhat higher rate than either Pen B or C. This difference was lost during the experimental feeding period, so that by Aug. 6 all the pens were laying at approximately the same rate.

3. There is not the slightest evidence, either from the figures presented above, or from the behavior or appearance of the birds during the experiment that the feeding of pituitary substance affected egg production in any way, either favorably or unfavorably. The results of this experiment thus confirm and extend the earlier work of this laboratory on pituitary substance.

4. Incidentally the figures and diagram show clearly the grave danger of error which inheres in drawing positive conclusions from minor fluctuations in an egg production curve, even in the presence of careful controls. Suppose, for example, that in the present instance the feeding of pituitary had begun about June 12, instead of on July 10 as was the fact. Immediately after June 12 the Pen A birds entered upon a period of heightened production, which was only to a small degree paralleled in the laying of the control pens B and C. One would have been very likely to conclude that this rise was due to the feeding of pituitary substance, had pituitary been fed at that time. Evidence of the favorable action of any agent on egg production needs careful scrutiny before it can be accepted. In particular the nature and amount of the change supposed to have been induced by the agent applied can be correctly and fairly interpreted only when account is

taken of the production curve of the experimental birds, and an equally large lot of control birds, over a preliminary period of at least 2 or 3 months. These precautions are often overlooked and quite erroneous conclusions reached by inexperienced students of egg production (see below).

II.

Another line of investigation which seemed worth undertaking was to determine whether the initial activation of the ovary could be accelerated by means of pituitary substance. A pullet of a good producing strain hatched at the proper time and well grown will ordinarily begin to lay when from 5 to 6 months of age. Some are more precocious and some less so, but, in general, the figures given represent a fair average. Can such pullets be brought to sexual maturity and laying any earlier if regularly fed pituitary substance?

To try this matter the following experiment was carried out. Forty-five pure-bred barred Plymouth Rock pullets, all hatched the same day (Apr. 29, 1915), were divided into three lots of fifteen each. They were so chosen that the total weight of the three lots, and thus the average weight per bird, were identical at the beginning of the experiment. Great pains were taken to get birds of the same stage of maturity and physiological development, as far as could be determined. Each bird in one lot (A) received *per os* 0.082 gm. per day of pituitary body (anterior lobe) substance. This was administered in No. 2 gelatin capsules with lactose as a diluent. Each bird in the second lot (B) received *per os* the same amount (0.082 gm.) per day of desiccated corpus luteum substance from pregnant cows, again administered in No. 2 gelatin capsules with lactose as a diluent. Finally Lot C was a control, the birds receiving no capsules. All the birds were housed in the same house, and given the same food and care except for the capsule feeding as above noted.

The experiment began Aug. 13, 1915. The growth of the birds is shown in Table II.

The essential facts brought out by this table are shown graphically in Fig. 2.

From the table and diagram it appears that:

1. Both pituitary body (anterior lobe) substance and corpus luteum substance retarded the growth of the birds in this experiment. Throughout the experiment all the birds were in a perfectly healthy and active condition. It was impossible to detect any difference in this respect among the three lots. There appears to be no means of accounting for the growth differences observed except as a result of the organ substances fed.

2. The retardation of growth in the case of the birds fed corpus luteum substance (Lot B) is greater than that in the lot fed pituitary substance (Lot A). The differences are very small and not separately significant until the weighing of Sept. 11 and later, from which time they are relatively large.

TABLE II.

Showing the Weights at Various Intervals of Birds Fed Pituitary Body
(Anterior Lobe) Substance and of Those Fed Corpus
Luteum Substance.

Date.	Lot A. (Pituitary.)		Lot B. (Corpus luteum.)		Lot C. (Control.)	
	Total weight.	Mean weight per bird.	Total weight.	Mean weight per bird.	Total weight.	Mean weight per bird.
	kg.	*gm.*	*kg.*	*gm.*	*kg.*	*gm.*
Aug. 13. Initial weight.	16.78	1,112.2	16.78	1,112.2	16.78	1,112.2
" 28	22.09	1,472.6	21.91	1,460.6	22.14	1,475.7
Sept. 4	23.81	1,578.6	23.59	1,571.1	24.18	1,611.8
" 11	24.68	1,645.0	24.22	1,614.8	25.40	1,693.4
" 18	25.72	1,714.6	25.45	1,696.4	26.81	1,787.2
" 25	27.12	1,808.3	25.63	1,708.5	28.26	1,883.9

3. After 43 days of organ substance feeding of birds starting at the same body weight, the birds fed pituitary substance average 4.01 per cent less in body weight, and those fed corpus luteum substance average 9.31 per cent less in body weight, than normal controls.

A parallel experiment to that reported above has been run with male birds and gave essentially the same results. Since the numbers used were too small to give reliable figures they are not published.

Leaving now the matter of growth in body weight we may turn to a consideration of the attainment of sexual maturity in these three lots of birds. In handling the birds for weighing
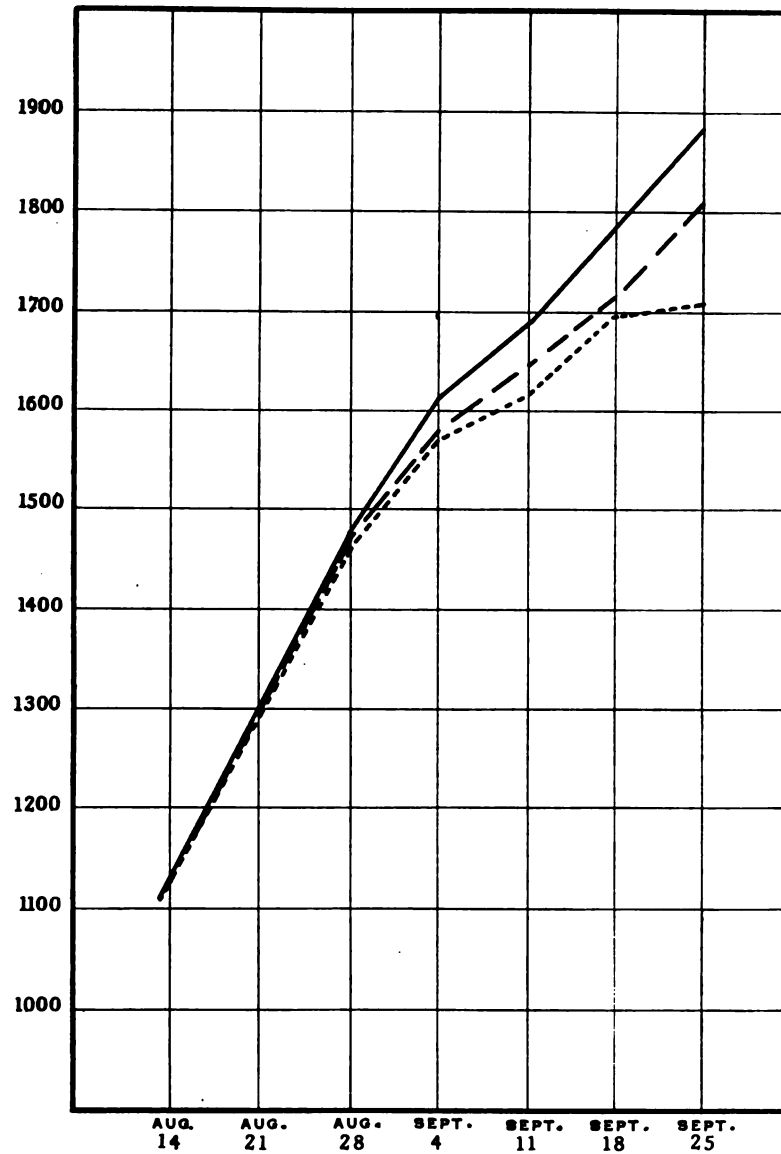
FIG. 2. Diagram showing mean weight per bird at successive dates following the feeding of pituitary substance and corpus luteum substance. Solid line, Lot C (control); broken line, Lot A (pituitary); dotted line, Lot B (corpus luteum).

and making general observations upon them no essential differences were detected.

The facts regarding onset of egg laying activities are shown in Table III.

From this table it is evident that there was practically no difference in the three lots of birds in respect to time of beginning laying. The small differences exhibited cannot be regarded as significant. Whether there would be a difference in average egg production in the several lots over a long period of time cannot be told from the present data, as the experiment had to be ended on Sept. 26.

TABLE III. .

Showing Beginning Egg Production of Birds Fed Different Organ Substances as Indicated.

Date.	Eggs laid by		
	Lot A. (Pituitary.)	Lot B. (Corpus luteum.)	Lot C. (Control.)
Sept. 16	—	1	—
" 17	—	1	—
" 18	1	1	—
" 19	1	1	—
" 20	1	1	—
" 21	—	—	1
" 22	1	—	—
" 23	—	—	1
" 24	—	—	1
" 25	—	1	—
" 26	1	. 1	1

Several interesting points are brought out by this experiment. In the first place the results with pituitary body substance confirm all our earlier work with this substance as far as activation of the ovary is concerned. There is no evidence that the administration of pituitary substance hastened in any way the initial activation of the pullet ovary. The pituitary birds did not in fact begin to lay so soon as those receiving corpus luteum substance, though the 2 day difference between the lots cannot be regarded as significant. We have now tested the effect of pituitary (anterior lobe) substance on the ovary in three different physiological states; viz., (a) completely resting adult ovary dur-

ing molt, (b) adult ovary in laying condition but with declining fecundity[4] rate, and (c) the inactive, immature ovary of the young pullet. In none of these physiological states has there been the slightest evidence that the pituitary has activated or accelerated the activity of the ovary in any manner or degree.

In the second place it appears that both pituitary (anterior lobe) substance and corpus luteum substance retard growth in the chick, but without affecting the attainment of sexual maturity (egg laying). The pullets in Lots A and B began to lay at the same time they would have had they not received organ substance, but their body weight at the onset of laying was from 4 to 9 per cent smaller than it would normally have been. These results are of interest in connection with the experiments of Gudernatsch[5] in feeding thyroid and thymus substance to growing tadpoles, though there is no evidence in the present experiments of differentiation being accelerated. It merely is not retarded, while body growth is retarded by pituitary and corpus luteum substance. It will be a matter of much interest to extend the period of feeding these substances, particularly corpus luteum, into earlier life. The writer proposes to do this next year. For students of growth a means is afforded in corpus luteum feeding of notably retarding body growth without disturbing the normal physiology or physiological development.

The results on growth in Lot A confirm the findings of various other workers who have studied the effect of pituitary substance on growth in other animals than the chick. Cushing[6] found that repeated injections of anterior lobe material caused a definite loss of weight. Sandri[7] found in mice and guinea pigs that pituitary feeding, or administration of an emulsion of the gland by injection, resulted in diminished growth. Aldrich,[8] apparently contrary to his expectations, found that when anterior lobe substance was fed to white rats it caused a definite and distinct re-

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[4] Compare Pearl, *Jour. Exper. Zool.*, 1912, xiii, 155, for definition of "fecundity" as used in papers from this laboratory.

[5] Gudernatsch, J. F., *Arch. f. Entwcklngsmechn. d. Organ.*, 1912-13, xxxv, 457; *Am. Jour. Anat.*, 1913-14, xv, 431.

[6] Cushing, H., *Jour. Am. Med. Assn.*, 1909, liii, 249.

[7] Sandri, O., *Arch. ital. di biol.*, 1909, ii, 337.

[8] Aldrich, T. B., *Am. Jour. Physiol.*, 1912-13, xxxi, 94.

tardation of growth. He fed 30 mg. per day for a period of 100 days and at the end of the period the body weight of the experimental rats was, on the average, 16.45 per cent in defect of that of the controls. The posterior lobe of the gland produced no effect on growth. Schäfer[9] appears to have been the only worker who has failed to find the retarding effect of anterior lobe substance upon growth, and his results have not been confirmed by subsequent workers.

I am not able to find that the similar but much more marked retarding effect of corpus luteum substance upon growth has hitherto been noted in the literature. Sack[10] has studied in rats the effect on general metabolism of feeding corpus luteum substance, and reports the finding of specific effects in the female. He has no data upon growth, however, and the period of feeding was so short and the number of animals used so small as to make his conclusions doubtful.

III.

While the investigations reported in this paper were in progress a paper by Mr. L. N. Clark[11] appeared dealing with the effect of pituitary body substance upon egg production in the domestic fowl. This author reports striking positive results in the direction of increasing egg production, following the feeding of anterior lobe. He attributes the difference between his results and those of Pearl and Surface to the fact that he used the gland substance of young animals while we used that from adults.[12] Whether or not the age of the animal furnishing the gland accounts for the difference in results between Mr. Clark and this laboratory remains to be shown. Pending the results of investigation directed towards elucidating that point, however, the present writer desires to express the opinion that Mr. Clark's figures do not appear to demonstrate completely his conclusion that the pituitary substance stimulated the ovary. Before this conclusion

[9] Schäfer, E. A., *Proc. Roy. Soc., Series B*, 1909, lxxxi, 442.

[10] Sack, W. T., *Arch. f. exper. Path. u. Pharm.*, 1912, lxx, 293.

[11] Clark, L. N., *Jour. Biol. Chem.*, 1915, xxii, 485.

[12] Mr. Clark's assumption that we used a commercial preparation is gratuitous. The material was especially prepared for us by Dr. Frederic Fenger.

could be accepted by anyone inclined to be critical it would be necessary to show by careful analysis of the egg production curves of the flocks used, extending over several months at least, that the fluctuations observed are not such as would have been as likely as not to occur had no experimental substance been fed. Mr. Clark's figures for his experimental pens contain no fluctuations greater, more sudden in onset, or more prolonged in duration, than the writer has repeatedly observed in the Maine Station flocks, *not subjected to any experimental treatment whatever at the time.* If the critical reader will study Fig. 1 of this paper, and then go over Mr. Clark's figures, plotting them as percentage productions so as to get experimental and control pens to the same base, he will get some idea of the extreme difficulty of adducing proof, or anything like proof, that a change upward in an egg production curve following the administration of an experimental agent is caused by that agent. Mr. Clark from his experience as a poultryman recognizes some of these difficulties, and properly points out that such experiments can only be considered critical when the general trend of the production curve is declining (p. 486).

One further point in which Mr. Clark's experiments appear to me to be open to serious criticism is in the fact that nowhere does he have any proper controls. In the first experiment where the thirty-five birds were fed individually flocks of 657 and 431 are cited as controls. But anyone who has had experience in experimental work with poultry knows that a reliable control can only be had by using a flock of the same size as the experimental flock, in which the individual birds are carefully selected so that they shall be as nearly as possible like the experimental birds in respect to all their characteristics including breed, age, body weight, stage of maturity, physiological condition, health, disposition, etc. Mr. Clark, as a practical poultryman, must surely appreciate these points, and as this first paper of his is said (p. 491) to be only preliminary, we may in his future experiments confidently look for more careful and critical attention to these safeguards which are essential to make a poultry experiment trustworthy. We shall look forward with great interest to the results of Mr. Clark's further and more critical experiments. Certainly the conclusions which he reaches are of great theoretical and practical interest, especially if confirmed by further critical work.

SUMMARY.

1. Feeding the desiccated substance of the anterior lobe of the pituitary body of cattle to hens in laying condition but at a time of year when the rate of fecundity is declining, does not stimulate the ovary to an increased rate of production.

2. Feeding the same substance (pituitary body, anterior lobe) to growing pullets does not bring about any earlier activation of the ovary than occurs in normal control pullets not fed this substance.

3. The anterior lobe of the pituitary body from cattle when fed to growing chicks is accompanied by a distinct retardation in growth in body weight. This confirms for the chick the results which have been obtained with this substance by other investigators in mammals.

4. The feeding of the desiccated substance of corpus luteum brings about a retardation of growth about twice as great in amount as that following pituitary feeding, as above noted.

5. Neither pituitary substance (anterior lobe) nor corpus luteum substance when fed to laying pullets causes any retardation in the attainment of sexual maturity as indicated by the laying of eggs. The birds so fed begin to lay eggs at the same age, but at a smaller body weight than the normal controls.

6. Some of the essential safeguards for the critical conduct of organ substance experiments with poultry are discussed.

CHANGES IN THE WEIGHT AND COMPOSITION OF · FASTING LOBSTERS.[1]

By SERGIUS MORGULIS.

*(From the United States Fisheries Biological Station, Woods Hole, and the
Biochemical Laboratory of the College of Physicians
and Surgeons, Columbia University.)*

(Received for publication, December 20, 1915.)

A statement in the literature[2] that lobsters retain their original
weight even after several months of fasting led to the present
investigation. The constancy of body weight may be regarded
as an index of physiological equilibrium, and its fluctuations indi-
cate the internal condition of the organism. By the fall in body
weight the normal course of a fast may be followed from day to
day, just as normal growth may be studied by the periodic gains
of the organism.

As far as we know, all organisms, temporarily deprived of
nourishment, diminish progressively in mass, and this is registered
by changes in weight. The observation of the constancy of the
weight of fasting lobsters seemed, therefore, worthy of investi-
gation. The two sides of the problem, *i.e.*, the changes in weight
and composition of the fasting lobsters, have been studied
simultaneously.

Preparatory to weighing, the lobsters were wrapped in towels
and the remaining traces of moisture were wiped off thoroughly
from every portion of the body. Each animal was then placed
in an aluminum can in which it was carefully weighed to 0.01
gm. The can was then weighed without the lobster, as the ani-
mals frequently excreted a small quantity of fluid during the
weighing, and the weight of the lobster was calculated. The
lobsters were kept in flat glass dishes partially filled with filtered

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[1] Published by permission of the Commissioner of Fisheries.

[2] Moore, B., Edie, E. S., and Whitley, E., *Report on the Lancashire Sea-
Fisheries*, 1913, xxii, 297.

sea water, which was continuously aerated. The water was changed every second day, and the weighing was made every 2 weeks. The experiment lasted for nearly 2 months.

The data relating to the changes in total body weight are recorded in Table I, in which the percentage of the biweekly change and the per cent of the loss suffered in the 56 days of inanition are given. It will be observed that the losses ranged from 0.10 to 1.89 per cent during the 2 week periods. On two occasions a slight increase of 0.3 per cent was observed. It will be seen from the table that all the lobsters lost weight, though this loss was extremely small. Thus in the 56 days of fasting they lost, on an average, 4.83 gm. or 2.89 per cent of their initial weight. In view of the facts recorded here it is hardly justifiable to speak of a constancy of weight of lobsters subjected to inanition. The question naturally arises as to whether the singularly low rate of loss of body weight indicates that the metabolic activity of these animals is very low, or that the actual loss sustained by the lobster is masked by secondary occurrences. The study of the composition of normal and starved lobsters gives a definite answer to this question.

The following methods have been employed in the investigation of the chemical composition. Both the control and experimental animals were rinsed with clean water, thoroughly dried, weighed, and after being cut .in small pieces they were desiccated at about 100°C. When no further diminution in weight was found, the dry material, shell and all, was ground to a fine powder which was again dried in the oven until the weight remained constant. The dry residues from the different lobsters were combined, thoroughly mixed, and used for the various analyses. The determinations made on these composite samples were as follows: total ash, non-volatile inorganic portion, total nitrogen, glycogen, and the ether-, alcohol-, and water-soluble matter. In the latter two portions the nitrogen was likewise determined.

In determining the total amount of ash a weighed portion of the dry material was at first charred over a low flame. This preliminary heating was never carried further than the incipient dull redness. The charred material was repeatedly extracted with boiling water until the test for chlorides was negative. The extracted material was then completely incinerated while the extract was evaporated. The combined weight of the two is the total ash content. The non-volatile inorganic constituents were determined separately by incinerating the dry matter directly in a muffle.

The glycogen was isolated according to Pflüger, and after hydrolysis it was measured by Allihn's gravimetric method.

TABLE I.

Changes in Weight of Lobsters Fasting from July 12 until September 6.

No.	1		2		3		4		5		6		Average	
Date.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.	Weight.	Bi-weekly loss.
	gm.	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*
July 12	175.50		168.33		143.87		189.85		165.15		162.35		167.51	
" 26	176.11	+0.35	167.29	−0.62	143.03	−0.60	189.20	−0.40	163.56	−0.97	159.69	−1.70	166.48	−0.62
Aug. 9	173.62	−1.42	165.67	−0.97	142.89	−0.10	187.85	−0.71	161.18	−1.46	159.31	−0.24	165.09	−0.84
" 23	173.19	−0.25	165.35	−0.20	141.35	−1.08	187.55	−0.16	161.72	+0.34	158.18	−0.80	164.56	−0.32
Sept. 6	171.31	−1.08	163.17	−1.32	138.68	−1.89	186.18	−0.73	160.77	−0.59	155.97	−1.39	162.68	−1.14
Total loss	4.19	−2.39	5.16	−3.07	5.19	−3.61	3.67	−1.94	4.38	−2.65	6.38	−3.93	4.83	−2.89

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The extraction with ether, then with alcohol and water, was made on the same sample of dry material, of which about 20 gm. were used for each determination. We used ether redistilled over sodium, and the alcohol was prepared from absolute alcohol by boiling it for 2 days with lime and distilling it over lime. This alcohol was kept over anhydrous copper sulphate.

The extraction with alcohol, which followed the ether extraction, was made in the same Soxhlet apparatus. By wrapping the apparatus with a layer of cotton it was possible to raise the temperature of the extraction cup so that a continuous flow of hot alcohol was maintained for 48 hours.

The material thus treated with ether and alcohol was boiled several times with distilled water until the filtered extract was perfectly colorless. The combined watery extracts were condensed and made up to 200 cc. Aliquot portions of this served for the nitrogen determination, as well as for the organic residue. This was determined by evaporation to dryness and analyzing the residue for its total yield of ash by the above method. The amount of organic extractives was obtained by subtracting the weight of the ash from the total dry weight.

Table II consists of two parts: in one the percentage composition of the dry matter of both normal and fasting lobsters is given; in the other these data are recomputed for the fresh material. Normal lobsters have on an average 67.33 per cent of water, the individual variations ranging from 65.9 to 69.5 per cent. About two-thirds (64.3 per cent) of the dry matter is organic. Of the inorganic matter about three-fourths is non-volatile constituents, while the volatile material consists chiefly of chlorides. The ether, alcohol, and water extractives represent 2.92, 5.61, and 10.53 per cent of the dry matter, respectively. The alcohol-soluble moiety contains 7.9 per cent of nitrogen, while in the water extractives there is 10.89 per cent of nitrogen. In determining the protein content I have departed from the customary way of giving as protein the product of the total nitrogen by the factor 6.25, but the nitrogen extracted by the various solvents which is not of protein origin has been deduced from the total amount of nitrogen found in the dry material. From the results of our experiments we may safely say that Moore is probably wrong in stating that "there is very little nitrogen other than protein nitrogen."[1] In my experiments the alcohol- and water-extracted nitrogen forms 22 and 24 per cent, respectively, of the total nitrogen of fasting and normal lobsters.

With the correction as stated above, the protein content of normal lobsters is computed as 31.54 per cent. But even this figure

TABLE II.

Percentage Composition of Normal and Fasting Lobsters (Fresh).

	Dry matter.	Water.	Organic matter.	Total inorganic matter.	Non-volatile inorganic matter.	Total glycogen.	Ether extract.	Alcohol extractives.	Alcohol-extracted nitrogen.	Per cent of nitrogen in alcohol extract.	Water extractives.	Water-extracted nitrogen.	Per cent of nitrogen in water extract.	Total nitrogen.	Protein (non-extracted nitrogen × 6.25).	Undetermined.
Control (average of five)	32.672	67.328	21.010	11.662	8.955	0.161	0.954	1.831	0.145	2.582	3.285	0.374	3.558	2.168	10.315	4.464
Fasting 56 days (average of five)	21.374	78.626	10.766	10.608	8.938	0	0.051	0.361	0.019	1.100	1.248	0.171	2.924	0.957	4.793	4.313

Percentage Composition of the Dry Matter of Normal and Fasting Lobsters.

	Organic matter.	Total inorganic matter.	Non-volatile inorganic matter.	Total glycogen.	Ether extract.	Alcohol extractives.	Alcohol-extracted nitrogen.	Per cent of nitrogen in alcohol extract.	Water extractives.	Water-extracted nitrogen.	Per cent of nitrogen in water extract.	Total nitrogen.	Protein (non-extracted nitrogen × 6.25).	Undetermined.
Control (average of five)	64.305	35.695	27.409	0.494	2.919	5.605	0.443	7.904	10.528	1.146	10.891	6.636	31.544	13.215
Fasting (average of five)	50.370	49.630	41.817	0	0.238	1.691	0.087	5.145	5.767	0.876	15.190	4.475	22.425	20.249

is probably too high, since some of the nitrogen considered as belonging to the protein is really a component part of the chitin of which the shell is largely made up. Chitin contains about 6 per cent of nitrogen (protein contains 16 per cent). Unfortunately the weight of the shells was not determined, and it is therefore impossible to compute even approximately the chitin nitrogen fraction. But it is clear that the amount of protein is less than that given in the table and that the undetermined portion, recorded in the last column, should be correspondingly higher than 13.2 per cent.

The composition of fasting lobsters, as compared with that of the normal lobsters, shows an unusually large increase in the proportion of water, which was increased to 78.63 per cent, with individual variations ranging from 77.6 to 79.7 per cent. An increased content of water has been generally observed in fasting organisms, but the change from 67.3 to 78.6 per cent, i.e., by more than 11 per cent, must be regarded as entirely unparalleled in any other animal. The dry matter is made up of practically equal parts of organic and inorganic substance. About four-fifths of the latter is non-volatile, a proportion greater than that found in normal lobsters, which is due to the predominant loss of chlorides and possibly also carbonates. The ether-, alcohol-, and water-soluble materials show likewise a marked reduction to 0.24, 1.69, and 5.77 per cent, respectively. The diminution is particularly noticeable in the case of the ether-soluble substances. the quantity of which decreased to less than one-twelfth the normal content. In comparison with that, the alcohol and water extractives have diminished much less, the former from 5.6 to 1.7 per cent, and the latter from 10.5 to 5.8 per cent. The nitrogen content of these extractives has not diminished in the same ratio. In the alcohol extractives it has changed from 7.9 to 5.1 per cent, while in the water extractives it increased from 10.9 to 15.2 per cent, or nearly one and a half times. It follows, therefore, that in the fasting lobsters the non-nitrogenous fraction of the extracted material decreased more rapidly than the nitrogenous.

It will be observed, furthermore, that the glycogen has been exhausted so that in the samples analyzed, at any rate, nothing could be recovered. It is possible, of course, that a slight trace may still have been present in the entire lobster.

The undetermined moiety of the dry matter of fasting lobsters is 20.25 per cent, and this is considerably higher than in the normal animal. It has already been pointed out that the undetermined fraction is doubtless chitin, and this would suggest further that the percentage of chitin is greater in fasting lobsters, which may be due either to the fact that the amount does not diminish as rapidly as that of the proteins, or that it does not diminish at all. In this connection the per cent of undetermined material found in the fresh substance gains particular significance. It will be seen that this was 4.46 per cent in the normal and 4.31 per cent in the fasting animals. We shall return to this subject later on.

The analyses here recorded differ considerably in their results from those given by Moore and Herdman.[3] This may be partly accounted for by the consideration that these authors determined only the total nitrogen and, multiplying by the usual factor 6.25, expressed it in terms of protein. But apart from this they found in their lobsters a much higher proportion of fat, glycogen, and ash. Even in the series which fasted 235 days they still found 2.14 per cent of fat, whereas my lobsters contained barely over 0.2 per cent at the end of 56 days. The per cent of undetermined material is, therefore, much lower than in my experiments. But here again, since Moore and Herdman consider all the nitrogen of the dry matter of lobsters to be protein nitrogen and do not make any allowance for the chitin, it is obvious that the actual amount of undetermined matter must be greater than is shown in their tables.

With the data given in Table II we may now compute the average composition of the lobsters before and after the fast, in terms of the absolute quantities of the various components. This has been done in Table III. The interesting fact brought out by this comparison of the initial and final composition is that the water in the organism is not merely increased relatively, as has been ordinarily found in the case of starving animals, but that it increased *absolutely*. Thus it is computed that at the start there were 112.67 gm. of water per animal. The amount actually

[3] Moore, B., and Herdman, W. A., *Report on the Lancashire Sea-Fisheries*, 1913, xxii, 321.

TABLE III.

Average Composition, in Grams, of Lobsters before and after Fasting.

	Live weight.	Dry matter.	Water.	Organic matter.	Total inorganic matter.	Non-volatile inorganic matter.	Total glycogen.	Ether extract.	Alcohol extractive.	Alcohol-extracted nitrogen.	Water extractive.	Water-extracted nitrogen.	Total nitrogen.	Protein (non-extracted nitrogen × 6.25).	Undetermined.	Per cent of organic matter determined.
Initial (computed)..	167.34	54.67	112.67	35.16	19.51	14.99	0.27	1.60	3.06	0.24	5.51	0.63	3.63	17.26	7.46	78.78
Final (determined)..	162.58	34.75	127.83	17.50	17.25	14.53	0	0.08	0.59	0.03	2.03	0.28	1.56	7.79	7.11	59.95
Absolute loss in gm..	4.76	19.92	+15.16	17.66	2.27	0.45	0.27	1.51	2.48	0.21	3.48	0.35	2.07	9.47	0.35	
Loss in per cent......	2.73	57.3	+13.46	50.22	11.62	3.03	100.0	94.80	80.84	87.20	63.17	55.59	57.11	54.75	4.69	

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found at the end of 56 days of starvation was 127.83 gm., or 15.16 gm. more.

We may also point out the comparative constancy of the non-volatile portion of the ash. It was diminished by 0.45 gm., or 3.03 per cent, and it is doubtful if such a small diminution has any significance in view of the fact that the initial quantity is computed and not directly determined. It is probably more nearly true to assume that the non-volatile inorganic constituents remain practically unchanged through fasting.

It should further be observed that the various extractives suffer most heavily. Thus 94.8 per cent of the ether-soluble material is used up, while of the alcohol and water extractives 80.8 and 63.2 per cent, respectively, disappear. It is significant that the amount of material which remained undetermined in our analyses is nearly the same in the lobsters before and after the fast. The loss of 0.35 gm., or 4.69 per cent, does not seem large enough to merit much consideration, and, though it would not justify the assumption that this particular component does not undergo any change, it is clear that the change must be of slight importance.

If the different organic constituents are added together it is found that their weight makes up 78.78 per cent of the total dry matter in the normal condition, and only 59.95 per cent in the starved. At first thought this failure to recover more of the organic substance in spite of the more or less extensive analytical treatment would suggest that the analyses were faulty. I have assumed this attitude towards the results of the analyses, but the same data were obtained by repeating practically the whole work. The question has been cleared up, however, by the fact that chitin, which is apparently unaffected by inanition, forms a larger proportion of the fasting than of the normal lobster. It is of interest to observe that a substance comparatively rich in nitrogen and calorific value should remain untouched, while under the great stress produced by the inanition all other tissues of the organism contribute heavily to its maintenance. It raises the problem of the availability of glucosamines as a source of energy. This point will be studied in the future.

We shall now attempt to answer the question we have undertaken to solve at the beginning of this paper. A review of the

data in the last line of Table III shows that as far as the different organic substances of the lobsters are concerned the rate of their loss of weight was as great as in any other known instance on record. The changes we find here are in close agreement, for instance, with those which I observed several years ago in starving salamanders.[4] The actual loss in body weight suffered by the lobsters is masked by the imbibition of water. We can easily compute what the probable loss was at the end of 56 days of fasting. The loss found from the change of the total body weight was only 2.73 per cent. But let us suppose that no imbibition of water had taken place. Furthermore, with the knowledge gained from other studies of fasting organisms we may assume that at this particular phase of starvation the quantity of water in the body would have diminished about 33.3 per cent. Starting with a quantity of 112.7 gm. of water, this would have decreased to 75.1 gm. at the end of 56 days of fasting. The weight of the lobsters would therefore have been 109.8 gm., and the loss 34.4 instead of 2.73 per cent. The hard shell protecting the entire body of the lobster and forming a solid supporting structure prevents the cells of the soft tissues from shrinking as they ordinarily do under the influence of inanition when the metaplasmic inclusions are being gradually used up. This may explain the extraordinary extent of the imbibition of water by the tissues as their reserves are being exhausted. The relative increase in the water content of the body which invariably occurs in inanition is unquestionably to reduce the concentration of the body juices. But the great absorption of water by the tissues of starving lobsters is the result of primarily mechanical factors, the tissues imbibing an excess of water in the manner of a sponge.

My thanks are due to Mr. E. W. Fuller for the assistance which he rendered in the course of these experiments.

[4] Morgulis, S., *Arch. f. Entwcklngsmechn. d. Organ.*, 1911, xxxii, 169.

THE LEWIS AND BENEDICT METHOD FOR THE ESTIMATION OF BLOOD SUGAR, WITH SOME OBSERVATIONS OBTAINED IN DISEASE.

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A large number of methods have been described for the estimation of blood sugar. Although many of these determine the reducing power of the blood with a high degree of accuracy, they are almost all so technical as to require special equipment and training. S. R. Benedict, who introduced the most satisfactory qualitative and quantitative tests we possess for sugar in urine,[1] has performed, in collaboration with Lewis,[2] a similar service for the sugar of the blood. The general principles and technique of this method are far simpler than those of any method previously described, and it seems probable that it will completely replace other blood sugar methods for both scientific and clinical purposes. The method is dependent upon the fact that very small amounts of glucose react with picric acid and sodium carbonate to produce a red color (picramic acid). In the actual test the picric acid serves the additional purpose of precipitating the proteins of the blood.

R. G. Pearce[3] has recently suggested a modification of the method to overcome the necessity for evaporation required with the original method. For more than a year and half we have utilized a procedure which accomplishes this purpose more simply, we believe, than that described by Pearce. It is the purpose of the present paper to present briefly the technique which we have

[1] Benedict, S. R., *Jour. Biol. Chem.*, 1909, v, 485; 1911, ix, 57.
[2] Lewis, R. C., and Benedict, S. R., *Jour. Biol. Chem.*, 1915, xx, 61. The paper contains an excellent review of the older methods.
[3] Pearce, R. G., *Jour. Biol. Chem.*, 1915, xxii, 525.

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employed, together with some of our observations on hyperglucemic conditions obtained with the method.

The Lewis-Benedict procedure calls for a blood dilution of 1: 12.5.   2 cc. of blood are treated with 8 cc. of water to lake the corpuscles and then 15 cc. of saturated picric acid are added to precipitate the proteins.   Pearce utilizes the same dilution, but to avoid evaporation, develops the color in the diluted solution with the aid of the higher temperature of the autoclave.   Comparison is made with a picramic acid solution one-fourth as strong as that utilized by Lewis and Benedict.

In connection with a rather large number of estimations of creatinine and creatine in the blood,[4] it was found advantageous to utilize the same preliminary step for the determination of blood sugar.   5 or 6 cc. of blood are treated in a 50 cc. centrifuge tube with four volumes of water to lake the corpuscles and dilute the blood.   To the mixture is then added sufficient dry picric acid (0.5 to 1.0 gm.) to precipitate the proteins and render the solution saturated.[5]   The mixture is stirred at intervals until it is a light yellow, then centrifuged, and the supernatant fluid filtered through a small filter paper.   10 cc. of the filtrate are employed for the estimation of creatinine, 5 cc. for creatine, and 3 cc. for sugar.   By employing 3 cc. of this filtrate from the blood diluted 1 : 5, the volume is sufficiently small to require no evaporation in the development of the color with the sodium carbonate. 3 cc. of this filtrate, the equivalent of 0.60 of 1 cc. of blood, are very close to the quantity employed by Lewis and Benedict— their 8 cc. are the equivalent of 0.64 cc. of blood.   With normal blood this contains a quantity of sugar sufficient to give a depth of color at a 10 cc. dilution most suitable for comparison in the colorimeter.   Heating a test-tube containing 3 cc. of the picric acid filtrate and 1 cc. of 20 per cent sodium carbonate in a beaker of boiling water for 15 minutes suffices for the development of the color, after which the tube is cooled and diluted to a volume suitable for colorimetric comparison.

[4] Myers, V. C., and Fine, M. S., *Jour. Biol. Chem.*, 1915, xx, 391.   Myers, V. C., and Lough, W. G., *Arch. Int. Med.*, 1915, xvi, 536.   A description of these methods has been given in Myers, V. C., and Fine, M. S., with the collaboration of Bailey, C. V., and Gorham, F. D., Chemical Composition of the Blood in Health and Disease, New York, 1915.

[5] Compare Folin, O., *Jour. Biol. Chem.*, 1914, xvii, 475.

In our earlier determinations the Duboscq colorimeter was employed, but more recently we have used the Hellige (Autenrieth and Koenigsberger) instrument, which is much more rapid and quite as satisfactory. As a standard color, picramic acid has been employed since Dr. Benedict suggested its use to us some months prior to the publication of his paper. We are further indebted to him for placing a supply of this chemical at our disposal.

Epstein[6] has suggested a modification of the Lewis-Benedict method whereby very small amounts of blood (0.2 cc.) are employed, this being obtained by puncture of the finger tip or lobe of the ear. Our experience has been that taking blood from a vein and thus securing a larger amount is more satisfactory and causes the patient less discomfort. Where the estimation of the blood sugar is not a part of a general blood examination in which 25 to 30 cc. are withdrawn, we have found the use of a 2 cc. glass barrel syringe fitted with a fine irido-platinum or steel needle to be very satisfactory. In this way blood has been taken from patients every 15 minutes over the greater part of a day.

Method.

About 2.5 cc. of blood are drawn into a glass syringe, which has been washed out with a drop or two of 20 per cent potassium oxalate to prevent clotting, and then delivered into a test-tube containing a very little powdered potassium oxalate. With an Ostwald-Folin pipette 2 cc. of the blood are pipetted into a cylindrical centrifuge tube of 15 to 20 cc. capacity graduated to 10 cc. The pipette is thoroughly washed out by pipetting 8 cc. of water into the tube. The blood is thus diluted 1: 5 and the corpuscles are thoroughly laked. About 0.2 gm. of dry picric acid is now added and the material thoroughly mixed with a glass stirring rod. After the protein is completely precipitated and the solution has become saturated with picric acid, it is allowed to stand several minutes with occasional stirring. The tube is now centrifuged and the supernatant liquid filtered into a dry test-tube through a small 4 cm. filter paper. 3 cc. (sufficient material is generally available for duplicate determinations, if de-

[6] Epstein, A. A., *Jour. Am. Med. Assn.*, 1914, lxiii, 1667.

sired) of the filtrate are pipetted into a tall, narrow test-tube, graduated to 3, 4, 10, 15, and 20 cc., 1 cc. of 20 per cent sodium carbonate is added, and the tube heated in a beaker of boiling water for 15 minutes. At this stage the color is completely developed though uninfluenced by prolonged heating. The solution is now cooled to room temperature and water added to bring the volume up to 10, 15, or 20 cc., etc., according to the depth of color. The solution is thoroughly mixed and compared in the Hellige colorimeter with the wedge of standard picramic acid.[7] The scale is most accurately read between 40 and 65. This would require a dilution to 10 cc. for normal blood. If the standard Hellige instrument is used with 0 at the top of the scale,[8] the value of the colorimetric reading in percentage of blood sugar may be obtained by subtracting the colorimetric reading from 100 and multiplying by the factor 0.002 if the dilution was to 10 cc., by 0.003 if the dilution was to 15 cc., etc.

In case it is desired to employ the Duboscq colorimeter this may readily be adapted to the above procedure by using a picramic acid solution which is the exact equivalent of the color of 0.6 mg. of glucose treated as above and diluted to 10 cc.[9] When the standard is set at 10 mm., a blood containing 0.10 per cent sugar manipulated as above with 10 cc. dilution, will match at 10 mm., since the 3 cc. of the picric acid filtrate employed are the equivalent of 0.6 cc. of blood. For the calculation the following formula, in which D represents the dilution and R the colorimetric reading may be employed, $\dfrac{D \times 0.1}{R}$ = blood sugar in per cent.

[7] This is prepared by dissolving 0.100 gm. of picramic acid and 0.2 gm. of anhydrous sodium carbonate in 30 cc. of warm distilled water and finally diluting to 1 liter.

[8] The scale has been inverted and dropped 2 mm. in the instrument modified by Rowntree for the phenolsulphonephthalein test. This scale may be made to correspond with the regular instrument by subtracting the reading found from 100 and adding 2.

[9] Glucose in picric acid solution keeps permanently. 3 cc. of 0.2 per cent glucose solution in saturated picric acid, treated with 1 cc. of 20 per cent sodium carbonate and heated as above may serve to standardize the picramic acid or, in fact, may be used as the standard itself. Such a solution is likewise valuable for use as an occasional control on the method. Although the picramic acid standard is practically permanent, it is best not to give it undue exposure to strong light. For the Duboscq the standard needs to be about two-thirds as strong as for the Hellige.

When only an occasional blood sugar estimation is to be made, evaporation according to the original Lewis-Benedict method is not a great hardship. More than half the time required may be saved, however, by utilizing the 1 to 5 dilution employed above. Theoretically, the higher dilution is to be preferred, but practically we believe that with the lower dilution the extraction of the sugar is sufficiently complete to be within the limits of accuracy with the method. It may be argued that the color is not completely developed without evaporation. Should there be a slight difference (and it is by no means easy to demonstrate), it would not impair the accuracy with the Hellige instrument, since the curve is plotted according to this technique. With a 10 cc. final dilution each division on the scale (1 mm.) represents a difference of 2 per cent. If carefully carried out we believe the accuracy can be made to fall within 1 to 3 per cent. In most observations on the human subject, however, we do not consider that it is advantageous or desirable to compute results beyond the second decimal place. Differences which do not fall within these two figures can possess no practical significance. Utilizing the technique described, we have found it possible to make quantitative recoveries of glucose added to blood. Our early observations (made on hot solutions) led us to believe that the creatinine of uremic bloods would appreciably influence the accuracy of the sugar determinations. The color produced by the creatinine fades so rapidly on cooling and diluting, however, that there is very little, if any, influence on the result.

If for any reason the use of the autoclave is found advantageous or desirable, a 1:5 dilution of the blood is to be preferred, since a more suitable color is obtained for colorimetric reading.

Considerable experience with the method described above has convinced us that it may advantageously be employed for many other purposes aside from the estimation of blood sugar.

Observations upon Hyperglucemia with Special Reference to the Influence of Renal Disease.

Some rather interesting observations on hyperglucemia in both diabetes and nephritis have been obtained with the above method on various hospital cases. On account of their theoretical interest and the practical value they show the estimation of blood

sugar to have, these observations bear brief discussion. That hyperglucemia exists in severe nephritis has been recognized for some time,[10] although a satisfactory explanation has not been advanced. The influence of the nephritis which generally accompanies advanced diabetes upon the threshold point of sugar excretion is likewise a topic of considerable interest. When the hyperglucemia is lowered by dietary restrictions, the blood sugar in certain of these cases does not return to normal even with the disappearance of sugar from the urine. Without doubt in many of these cases the threshold point of sugar excretion has been raised by the nephritis, and furthermore it is probable that the nephritis exerts its usual elevating influence upon the sugar content of the blood.

Most of our determinations on cases with no known cause for hyperglucemia have shown values between 0.09 and 0.11 per cent, in confirmation of Lewis and Benedict. There are a considerable number of hospital cases, however, which show blood sugars of 0.12 to 0.14 per cent. The observations tabulated below represent the more interesting results obtained from about 500 analyses.[11] As a rule, the samples of blood have been obtained in the morning before breakfast.

In Table I are shown some rather pronounced hyperglucemias encountered in severe cases of nephritis. The first two cases in this table were fatal cases of mercuric bichloride poisoning. The next group of six cases suffered from chronic interstitial nephritis with uremia and were all fatal except the last. Glucosuria was not noted in the cases of mercury poisoning or of those suffering from interstitial nephritis. The last group of two cases suffered from chronic parenchymatous nephritis, and both showed hyperglucemia with an almost constant mild glucosuria.

Pavy[12] and other older investigators, and recently McLean,[13] have attempted to show that there is a direct relation between

[10] Compare Bang, I., Der Blutzucker, Wiesbaden, 1913, 128; also Neubauer, E., *Biochem. Z.*, 1910, xxv, 284; Rolly, F., and Oppermann, F., *ibid.*, 1913, xlviii, 268; Hopkins, A. H., *Am. Jour. Med. Sc.*, 1915, cxlix, 254.

[11] Most of these cases were patients in the medical wards of the hospital and we are indebted to Professor Edward Quintard and other members of the Medical Staff for the opportunity of studying these cases.

[12] Pavy, F. W., Physiology of the Carbohydrates, London, 1894, p. 193.

[13] McLean, F. C., *Jour. Am. Med. Assn.*, 1914, lxii, 917.

TABLE I.

Hyperglucemia in Nephritis.

Group.	Case.	Age.	Sex.	Date.	Sugar of blood.	Urea N of blood per 100 cc.	Remarks.
		yrs.			per cent	mg.	
I	W. F.	25	♂	Nov. 16, 1914	0.18	182	Fatal cases of poisoning with mercuric bichloride.
	—	29	♀	Oct. 27, 1915	0.17	65	
II	W. O'C.	33	♂	Mar. 7, 1914	0.19	200	
				" 9, 1914	0.20	182	
	M. K.	42	♂	May 13, 1914	0.18	140	
	I. D.	17	♀	Dec. 21, 1914	0.19	134	
				" 30, 1914	0.19	170	
	E. M.	39	♂	Nov. 30, 1915	0.18	97	
				Dec. 7, 1915	0.18	129	Cases of chronic interstitial nephritis with uremia, all fatal except the last.
				" 10, 1915	0.22	132	
	T. D.	34	♂	Jan. 22, 1915	0.09	60	
				Feb. 6, 1915	0.12	72	
				" 12, 1915	0.14	66	
				Mar. 9, 1915	0.14	97	
				" 16, 1915	0.14	120	
	J. W.	34	♂	Feb. 24, 1915	0.14	55	
				Mar. 1, 1915	0.10	63	
				" 6, 1915	0.10	89	
				Apr. 15, 1915	0.14	144	
	L. P.	57	♂	Jan. 6, 1915	0.14	80	
				Feb. 18, 1915	0.10	59	
				Mar. 1, 1915	0.10	37	
				" 5, 1915	0.10	45	
III	C. M.	50	♂	Apr. 15, 1915	0.20	15	
				" 27, 1915	0.19	16	Cases of chronic parenchymatous nephritis generalized edema high blood pressure large amounts of albumin and many casts in urine; low phthalein outputs; almost constant mild glucosuria, generally under 1 per cent.
				Dec. 7, 1915	0.17	11	
	J. P.	62	♂	July 27, 1915	0.18	28	
				Aug. 31, 1915	0.18	23	
				Sept. 24, 1915	0.17	25	
				Oct. 1, 1915	0.14	25	
				" 26, 1915	0.14	29	
				Nov. 19, 1915	0.16	28	
				Dec. 3, 1915	0.18	25	

TABLE II.

Influence of Nephritis upon the Excretion of Sugar in Diabetes.

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Severity of nephritis.	Remarks bearing upon nephritis.
				per cent	*per cent*		
M. G.	63	♀	June 3, 1914	0.19	0	++	Phthalein output 30 per cent; blood pressure (systolic-diastolic) 185–100; small amount of albumin and few casts in urine; no edema.
F. R.	47	♂	Feb. 17, 1915	0.22	0	++	Blood pressure 190–100; trace of albumin and few hyaline and granular casts constantly present in urine.
C. L.	57	♀	" 18, 1915	0.24	5.0	++	Trace of albumin and hyaline and granular casts in urine; endarteritis obliterans; arteries markedly sclerosed.
E. S.	27	♀	May 15, 1915	0.31	6.0	+	Trace of albumin at times; few hyaline casts; polyuria; no edema, headaches; blood pressure 95-60.
M. McC.	61	♀	Apr. 15, 1915	0.33	0.5	++	Blood pressure 190–110; urine small in volume; many casts; large amount of albumin; endarteritis obliterans.
S. F.	61	♂	Nov. 20, 1915	0.33	6.3	+	Few hyaline casts; albumin from prostatitis.
A. M.	68	♂	" 6, 1915	0.34	0	+++	Large amount of albumin; many granular casts; blood pressure 160-90; died.

TABLE II—*Continued.*

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Severity of nephritis.	Remarks bearing upon nephritis.
				per cent	*per cent*		
O. U.	60	♂	Oct. 8, 1915	0.35	.Trace.	?	Diagnosis, carci-noma of head of pancreas; symptoms of obstruction of common duct 6 wks.; no albuminuria.
J. E.	23	♀	June 4, 1914	0.36	7.0		Clinic case; no nephritic symptoms.
R. J.	47	♂	Mar. 16, 1915	0.36	1.2	+++	Phthalein 36 per cent; moderate albuminuria, granular casts; blood pressure 200-100 headaches a n d fail ng eyesight.
M. W.	53	♂	Apr. 10, 1915	0.37	1.7	++	Phthalein 44 per cent; a l b u m i-nuria, blood picture of interstitial nephritis; only slight acetonuria; died.
C. R.	35	♀	Mar. 21, 1915	0.38	6.2	?	Diabetes 6 yrs.; amputation of left foot for gangrene.
P. F.	48	♀	June 4, 1914	0.39	2.2	++	Phthalein 42 per cent; moderate a l b u m i n uria; only slight acetonuria.
A. McD.	30	♂	Mar. 21, 1915	0.42	5.0	?	Occasional slight a l b u m i n u r i a; marked edema.
B. S.	46	♀	Aug. 26, 1915	0.42	3.6	+++	Excessive albuminuria; many granular casts; marked acidosis; died in coma.

TABLE II—*Concluded*.

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Severity of nephritis.	Remarks bearing upon nephritis.
				per cent	*per cent*		
J. C.	50	♀	Nov. 26, 1915	0.46	0	+++	Phthalein 34 per cent; occasional trace of albumin; few casts; blood pressure 190–130; diabetes 15 yrs.; sugar excretion 0–2.5 per cent.
M. McC.	56	♀	Feb. 12, 1915	0.57	8.0	+	Phthalein 52 per cent; trace of albumin in urine on admission; no e d e m a; blood pressure 176–105; carbohydrate tolerance 80 gm.
R. B.	15	♂	Mar. 10, 1914	0.79	8.7	+ .	Marked polyuria; considerable acidosis; occasional trace of albumin in urine; tuberculosis.
L. F.	52	♀	Oct. 29, 1915	0.80	2.2	++	Patient died day a f t e r observation; a l b u m i-nuria; nitrogen retention; see first case of next table.

the degree of hyperglucemia and glucosuria, a view well supported by their observations.

Table II gives the concentration of the sugar in both the blood and urine in nineteen severe cases of diabetes. Most of the observations were obtained before the patients had been placed upon a greatly restricted diet; *i.e.*, at a time most favorable to the development of hyperglucemia and glucosuria. An inspection of the table shows that most of these patients likewise suffered from nephritis of more or less severity. Although some cases

with definite nephritic symptoms retained the power of secreting
a urine of high sugar content, severe nephritis appears to reduce
markedly the permeability of the kidney for sugar; and it is only
when these latter cases are excluded from the above table that
there appears to be any relation between the hyperglucemia and
glucosuria. Most writers on diabetes mention the influence of
nephritis upon the permeability of the kidney for sugar. Al-
though the above table probably gives an exaggerated impression
of the occurrence of nephritis in diabetes, since the cases were those
needing hospital care, it is not believed that the importance of
this subject has hitherto been fully appreciated.[14] It is worthy
of note that a number of the above cases with reduced carbohy-
drate intakes, but with blood sugars above 0.2 per cent, showed
a disappearance of sugar from the urine. When the threshold
point of sugar excretion has been raised by a nephritis, the disap-
pearance of sugar from the urine or a low sugar excretion is a
poor guide to the glucemia. This would appear to be a factor of
considerable importance in the application of Allen's starvation
treatment in diabetes. In their most recent communications,
both Allen[15] and Joslin[16] refer to the estimation of blood sugar,
although they have not as yet drawn any conclusions as to its
value in this connection.

The data on three of the fatal cases of diabetes deserve more
detailed consideration. As shown in Table III, the blood sugar
content of the first two of these cases was very high, the second
determination on L. F. being slightly higher than any we have
found recorded, although several cases mentioned by von Noor-
den[17] are very close to this figure, 1.10 per cent. In one case he
noted a blood sugar of 1.01 per cent and emphasized that neph-
ritis was not present. Lépine[18] likewise reports a case with a
blood sugar of 1.06 per cent. In discussing the influence of
nephritis upon the permeability of the kidney for sugar, von

[14] Compare Liefmann, E., and Stern, R., *Biochem. Z.*, 1906, i, 299; also
Rolly, F., and Oppermann, F., *ibid.*, 1913, xlix, 278.

[15] Allen, F. M., *Am. Jour. Med. Sc.*, 1915, cl, 480.

[16] Joslin, E. P., *Am. Jour. Med. Sc.*, 1915, cl, 485.

[17] von Noorden, C., Metabolism and Practical Medicine, English edition
by Hall, London, 1907, iii, 532.

[18] Lépine, R., La Diabète Sucré, Paris, 1909, 454.

Noorden writes: "I have myself drawn attention to an observation on a diabetic patient who died from uremic coma (not diabetic coma). The urine contained 1.4 per cent of sugar and the blood at the same time, 0.85 per cent."

The chemical blood pictures of the cases in Table III are typical of interstitial nephritis;[19] *viz.*, high uric acid values with more

TABLE III.

Composition of Blood in Three Fatal Cases of Diabetes.

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Uric acid.	Urea N.	Crea- tinine.	CO_2 — combining power (Van Slyke). Per 100 cc. of plasma.
							Per 100 cc. of blood.		
			1915	*per cent*	*per cent*	*mg.*	*mg.*	*mg.*	*mg.*
L. F.*	52	♀	Oct. 29	0.80	2.2	10.5	55	2.1	72
			" 30	1.10	0.5				61
M. W.**	53	♂	Apr. 10	0.37	1.7	6.0	18	2.0	
			" 17	0.98	1.6				
B. S.†	46	♀	Aug. 26	0.42	3.6	7.6	28	4.7	21

* Duration of disease, several years; no coma until day of death on Oct. 30. Small amount of albumin in urine of Oct. 26; 2.7 per cent of sugar; only faint trace of acetone in urine.

** Gangrene of toes at times during the last 5 yrs.; glucosuria very severe early in disease but later improved; systolic blood pressure 150-165; phthalein output 44 per cent; moderate amount of albumin in urine of Apr. 10; at death (Apr. 18) urine showed only small amount of acetone; uremic symptoms 30 hrs. before death and about 6 hrs. previous to the last blood analysis.

† Patient entered hospital in coma and died several hours later; urine contained very large amounts of albumin, acetone, and diacetic acid, and many granular casts.

or less increase in the urea. In the first two cases the creatinine was normal, although in the last case there was marked retention. The cause of death in the first two cases is obscure. It would scarcely appear to have been due to acidosis. The last case, however, showed marked acidosis, as ascertained from both the

19 Compare Myers, V. C., Fine, M. S., and Lough, W. G., *Proc. Soc. Exper. Biol. and Med.*, 1915, xiii, 5; see also von Noorden, *loc. cit.*, 669.

urine and the CO_2-combining power of the blood (Van Slyke). In this case the marked nephritic symptoms, coupled with the high creatinine, would seem to indicate that the nephritis had as much to do with the cause of death as the diabetes.

TABLE IV.

A Comparison between Diabetes Mellitus and Renal Diabetes.

Case.	Age.	Sex.	Date.	Sugar of blood.	Sugar of urine.	Diet.	Remarks.
			1915	*per cent*	*per cent*		
			May 15	0.31	6.0	50 gm. carbo-hydrate.	Severe diabetes; much emaciated; no edema; nervous symptoms; mild nephritis as evidenced by occasional trace of albumin in urine and few hyaline casts.
			" 17	0.30	1.5	}	
E. S.	27	♀	" 19	0.32	1.5	Hunger and whiskey.	
			" 25	0.28	0.2		
			June 1	0.17	0		
			" 8	0.27	0.6	15 gm. carbo-hydrate.	
			Aug. 3	0.09	1.0	} Hunger.	Probable "renal diabetes;" constant glucosuria for 7 yrs. in spite of treatment; at time of examination 4 mos. pregnant; no edema; greatly emaciated; thyroid slightly enlarged; blood pressure 95–60; trace of albumin and few finely granular casts in urine; phthalein 39 and 42 per cent.
			" 5	0.11	1.6		
			" 11	0.10	3.1	Green vege-tables.	
M. B.	31	♀	" 25	0.09	1.3	15 gm. carbo-hydrate.	

The concentration of blood sugar is probably more important in the recognition of non-diabetic conditions than diabetes proper. In Table IV are given observations on a case of "renal diabetes" and for comparison somewhat similar observations on a case of diabetes mellitus. With the latter considerable difficulty was encountered in obtaining a disappearance of sugar from the urine,

even with complete starvation (and here hyperglucemia was still present). The case appears similar to one recently reported by Joslin, although probably not as severe. The blood sugar values in M. B. were essentially normal. Neither the sugar of the blood or urine was especially influenced by dietary restrictions. Although "renal diabetes" is not an uncommon condition in pregnancy,[20] the history of the case stamps it as true "renal diabetes." At about the same time our attention was directed to a number of other pregnant women who excreted very small amounts of sugar. Application of the phenylhydrazine test, according to Cole,[21] showed the sugar to be lactose in every case.

DISCUSSION AND SUMMARY.

The Lewis-Benedict method for the estimation of blood sugar may be greatly simplified by employing an initial blood dilution of 1: 5 instead of 1: 12.5. In this way a sufficiently low concentration is obtained to obviate the necessity for evaporation in the development of the color. For this determination the Hellige colorimeter has been found especially satisfactory.

A number of cases of nephritis are reported with hyperglucemias close to 0.2 per cent. In the four cases of interstitial nephritis glucosuria was absent, while mild glucosuria was constantly present in the two cases of parenchymatous nephritis with edema. The blood sugar content of nineteen advanced cases of diabetes is reported together with the corresponding sugar excretion. Many of the cases gave evidence of nephritis, apparently of the interstitial type. If sugar was being excreted by these cases, the percentage output compared to the blood sugar was correspondingly lower than in other cases of diabetes. As recently emphasized by Mosenthal,[22] cases of interstitial nephritis secrete a urine of a very constant low specific gravity with low content of chloride and nitrogen. It is possible that this same factor may have some influence on the concentration of urinary sugar. A case is reported with 1.10 per cent of sugar in the blood and only

[20] Novak, J., Porges, O., and Strisower, R., *Z. f. klin. Med.*, 1913, lxxviii, 413.

[21] Cole, S. W., *Lancet*, 1913, clxxxv, 859.

[22] Mosenthal, H. O., *Arch. Int. Med.*, 1915, xvi, 733.

0.5 per cent in the urine. If the nephritis is of the interstitial type, the data obtained for uncomplicated nephritis explain the raising of the threshold point of sugar excretion in these advanced cases of diabetes. The nephritis may further explain the difficulty in reducing the blood sugar of these cases to normal by restrictions in the carbohydrate intake. The use of lactose as a functional kidney test has shown how quickly the permeability of the kidney is impaired for this sugar in nephritis. As an index of the ability of the kidney to excrete sugar, it seems possible that a ratio between the sugar of the blood and urine might be worked out somewhat after the method McLean[23] has recently employed for urea and chlorides.

Figures for the sugar of the blood and urine are also reported in a case of "renal diabetes."

[23] McLean, F. C., *Jour. Exper. Med.*, 1915, xxii, 212 and 366. Compare Ambard, L., Physiologie normale et pathologique des reins, Paris, 1914, 74.

IS AUTOLYSIS AN AUTOCATALYTIC PHENOMENON?

By MAX MORSE.

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As far as the writer is aware, no attempt has been made to determine the hydrogen or hydroxyl ion concentration of autolyzing digests. In an analytic study of the action of the enzymes involved, it is important to know the range of concentration of these components; for until the present time it has not been known what induces autolytic reactions, what changes the ionic concentration from a preponderance of hydroxyl ions to hydrogen ions, whether the hydrogen ion acts as a co-enzyme, activating the pro-enzyme of the tissues, or whether the process can be placed in the category of Ostwald's "autocatalysis,"[1] in which the process of enzyme action is accelerated by the products formed during the action of the enzyme and in consequence of it. From the pathological standpoint it is desirable to know the range of acidity developed in an atrophying tissue.

Wiener,[2] and Baer and Loeb[3] have studied the reaction of the medium in autolyzing digests, and the two reports are similar in respect to determinations of the range of acidity in which the enzymes of autolysis are active. Wiener finds that "bei einer Konzentration, die 0.2–0.4 per cent NaOH entsprach, die *Autolyse vollständig aufgehoben wird;*"[4] and that "*bei einer Alkaleszenz, wie sie der Blutalkaleszenz entspricht* und auch bei einer doppelt so hohen, *eine Autolyse* wenigstens durch eine Wochelang *ausgeschlossen ist.*"

Since the time of Hoppe-Seyler,[5] it has been customary to attribute the inception of autolysis to the development of products of intermediate oxidation, as the oxygen supply is reduced in cases involving autolysis *in vivo;* such compounds as lactic acid, etc., are known to be formed, and it is conceivable that the appearance of these compounds marks the beginning of autolysis. However, the writer has studied the rôle of oxygen in autolyzing tissue[6] with the result that no apparent effect seems to be produced

[1] Ostwald, W., *Lehrb. d. allg. Chem.*, 1910, ii, pt. ii, Verwandtschaftsl., 263.

[2] Wiener, H., *Zentr. f. Physiol.*, 1905–06, xix, 349.

[3] Baer, J., and Loeb, A., *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 1.

[4] Wiener, *loc. cit.*, 355.

[5] Hoppe-Seyler, F., *Med.-Chem. Untersuch.*, 1877, iv, 499.

[6] Morse, M., *Biochem. Bull.*, 1915, December (in press).

upon the rate of autolysis when oxygen gas or oxygen-liberating compounds are introduced into the digests. The fact that autolysis apparently proceeds in muscles where the blood system is intact, but the nerve supply interrupted,[7] indicates that the assumption which has been made concerning oxygen as the factor determining autolysis should be investigated. The rôle of starvation in inducing autolytic changes has been studied by some workers (Rettger,[8] for bacteria, etc.), where reduction in food induces autolytic processes. That autolytic enzymes are specific in some cases (Jacoby[9]) and heterolytic[9] in others has been the experience of other workers (Bradley[10]), so that it is difficult to explain the rise of autolytic changes in a given tissue in terms of the throwing off of the effects of any anti-enzyme, such, for instance, as Jobling[11] and his coworkers have found for trypsin in the unsaturated fatty acids and their soaps. In all the possible explanations of inception of autolysis, the only factor which seems common is the alteration in hydrogen ion concentration.   •

Bradley[10] has determined that an alteration probably occurs in the tissue substrate through the action of certain introduced reagents and perhaps through the action, likewise, of the acid normally produced during the process of autolysis. From the results presented here, it is evident that a relatively low hydrogen ion concentration is developed at the inception of the process and that the increase as autolysis proceeds is small and runs *pari passu* with the curve of soluble nitrogen. If the effect of acid production during autolysis were, as Bradley holds, of "two effects: (1) it will remove the inhibitory alkalinity, and (2) alter the proteins with a resulting increase in the mass of substratum,"[12] then we should imagine that the curve of the rate of autolysis in a normally digesting hash would show a latency at the beginning while the acid produced is being absorbed by the proteins, so that no free acidity is available for the reactions. Wiener[2] and Magnus-Levy[13] have recorded such a latency, but the present writer has not observed it in any digests, although the tissue was submitted to Salkowskian autolysis immediately after removal from the body. All curves of autolysis rate show[14] that the rise of curve is abrupt from the beginning, the greatest digestion proceeding within the first 12 hours of action. The only alternative to this conclusion is that the acid produced is so great that the tissue becomes saturated, with excess of acid. In this case, we should expect to have a sudden rise in acidity at a time when saturation of the proteins has been completed, representing the acid no

[7] Morse, *Am. Jour. Physiol.*, 1915, xxxvi, 147.

[8] Rettger, L. F., *Jour. Med. Research*, 1904-05, xiii, 79.

[9] Jacoby, M., *Beitr. z. chem. Phys. u. Path.*, 1903, iii, 446.

[10] Bradley, H. C., *Jour. Biol. Chem.*, 1915, xxii, 113.

[11] Jobling, J. W., and Petersen, W., *Jour. Exper. Med.*, 1914, xix, 239 ff.

[12] Bradley, *loc. cit.*, 123.

[13] Magnus-Levy, A., *Beitr. z. chem. Phys. u. Path.*, 1902, ii, 261.

[14] Compare Bradley and Morse, *Jour. Biol. Chem.*, 1915, xxi, 212 ff. Morse, *Jour. Biol. Chem.*, 1915, xxii, 125.

longer taken up by the proteins and thus remaining free for participation in the reaction. While such a condition is possible, we should seek the simpler explanation, that the acidity developed acts catalytically, activating the enzyme in much the same manner that the hydrochloric acid of gastric digestion activates; for there is nothing to indicate a sudden alteration in the H· content in the curves. The extent of digestion in an autolyzing hash does not require the assumption that the substrate has been affected so that more is present and capable of being digested; if the globulin portion is digestible, as Jacoby[15] determined and as the present writer has also found, the figures of Pohl[16] for liver globulin fraction are adequate to account for the digestion in autolysis without the assumption of increase of substrate. If any modification were to occur in the acid, it would be logical to assume that the conversion would tend towards globulin-like bodies, but the only case where a change of this nature has been reported has involved the action of alkali[17] and not of acids.

The method followed in the determination of the hydrogen ion concentration is briefly as follows: Thymus was used as substrate, owing to the colorless extract. Liver, which has been used in the writer's previous work on autolysis, cannot be used to advantage, the colorimetric determinations being rendered inexact by the retention of the reddish colors of the extracts. The method of preparation of the substrate was similar to that given in previous papers by the present writer. The preparation of the standard color solutions followed the method given by Sörensen[18] and in part the descriptions of Levy, Rowntree. and Marriott.[19] The following solutions were used:

Salt м/15.	Proportions in cc.														
	Methyl orange.						Phenolsulphonephthalein.								
P$_H$	4.5	4.9	5.3	5.6	5.9	6.2	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5
KH$_2$PO$_4$	10.0	9.9	9.75	9.5	9.0	8.0	7.3	6.3	5.1	3.7	3.2	2.7	2.3	1.9	1.58
Na$_2$HPO$_4$	0.0	0.1	0.25	0.5	1.0	2.0	2.7	3.7	4.9	6.3	6.8	7.3	7.7	8.1	8.42

The digests were removed for sampling and 10 cc. of the liquid were removed after shaking. The withdrawn liquid was placed in Schleicher and Schüll protein-impermeable dialyzing tubes.

[15] Jacoby, M., Ergebn. d. Physiol., 1902, i, 213.

[16] Pohl, J., Beitr. z. chem. Phys. u. Path., 1906, vii, 381.

[17] Moll, L., Beitr. z. chem. Phys. u. Path., 1904, iv, 562.

[18] Sörensen, S. P. L., Ergebn. d. Physiol., 1912, xii, 393.

[19] Levy, R. L., Rowntree, L. G., and Marriott, W. McK., Arch. Int. Med., 1915, xvi, 389.

which had previously been tested to insure that they were protein-impermeable, and the tubes were then placed in 50 cc. of distilled water. The control distilled water gave no color reaction with the indicators to warrant the preparation of conductivity water. The dialysis proceeded exactly 5 minutes, when the dialysate was removed to the extent of 3 cc., the amount in the standards. One drop of indicator was added, the preparation shaken thoroughly, and the colors were matched. Tubes of uniform size were used throughout (Wassermann tubes). The results are plotted in terms of the Sörensen nomenclature, P_H = log. conc. H·. Determinations were made at 12 hour intervals for the first 48 hours and thereafter at 24 hour intervals, all experiments being made in duplicate.
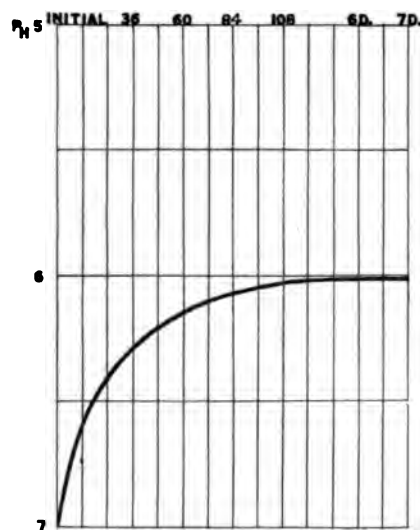
This curve is to be compared to the curves of autolysis rate in normal autolyzing hash.[20] The curves are similar in essentials. In both instances we find an abrupt rise, the greatest digestion, and the greatest development of acid occurring within the first 60 hours. As the curve of acidity approaches the plateau, the curve of digestion likewise reaches the maximum.[21]

[20] Compare references given above.
[21] There was no appreciable change in acidity after the fifth day.

It may be considered a debatable point whether the digestion is responsible for the similarity in the curves or whether it is a question of the effect of the acid. When we remember that the development of acidity is the *sine qua non* for autolysis, we have reason to believe that the process is an autocatalytic one, the developing acidity inducing greater and greater acceleration in the digestion rate, and when the acid reaches a maximum in its production, digestion likewise reaches its maximum.[22]

CONCLUSIONS.

1. Autolysis is an autocatalytic phenomenon, the products of digestion entering into the reaction as true catalyzers.

2. There is nothing in the study of acid production to indicate that the substrate is altered by the acid.

3. The present report substantiates studies of other investigators in regard to the degree of acidity necessary in autolysis: *e.g.*, Wiener, and Baer and Loeb; but no determination of acidity developed during digestion seems to have been made previously.

[22] Titrations with 0.05 N Ba(OH)$_2$, with thymolphthalein as indicator, yield results similar to those given by the colorimetric method. In this case protein-free filtrates were used. The curve is essentially similar to the one presented, which may be taken as the type of all curves concerned with the reaction of the medium in autolyzing digests.

THE OCCURRENCE OF p-HYDROXYPHENYLETHYL-
AMINE IN VARIOUS MISTLETOES:

By ALBERT C. CRAWFORD and WALTER K. WATANABE.

(*From the Division of Pharmacology, Stanford University Medical School, San Francisco.*)

In 1914 we reported[1] that *Phoradendron flavescens*, the mistletoe of our southeastern states, owed most, if not all of its pressor activity to p-hydroxyphenylethylamine. This raised the question whether this compound occurred in other species of mistletoe. In some of the work reported no distinction in varieties has been made, and this may lead to confusion. The classification of the genus *Phoradendron* is now under discussion.[2]

European workers have reported that *Viscum album*, the European mistletoe, will on intravenous injection into dogs cause a persistent fall in blood pressure with increase in urinary secretion. According to Selig, extracts of *Viscum album* increase the amount of urine secreted during the 24 hours. Extracts of this species of mistletoe have been introduced into therapeutics as a hypotensive agent.[3]

According to Chevalier,[4] *Viscum album* contains a volatile alkaloid, a resinous substance with a drastic action, and two glucosides. To these latter Chevalier ascribed the hypotensor and diuretic action. The volatile alkaloid was claimed to cause a transitory rise in blood pressure, stimulation of the medulla, and salivary and bronchial hypersecretion.

Barbieri[5] claims that the activity of *Viscum album* is not due to a saponin. While there has been much discussion as to the active compound in this plant, no one has yet isolated the hypotensor agent. Leprince[6] has obtained from this plant a volatile base having the composition $C_8H_{11}N$, but

[1] Crawford, A. C., and Watanabe, W. K., *Jour. Biol. Chem.*, 1914, xix, 303.

[2] Trelease, W., *Proc. Nat. Acad. Sc.*, 1915, i, 30.

[3] Selig, A., *Med. Klin.*, 1912, viii, 991.

[4] Chevalier, J., *Compt. rend. Soc. de biol.*, 1908, lxiv, 2. Lesieur, E., Contribution à l'étude du Gui, Thèse de Paris, 1910.

[5] Barbieri, O., *Arch. di farm. sper.*, 1913, xiv, 39.

[6] Leprince, M., *Compt. rend. Acad.*, 1907, cxlv, 940.

there is no report as to whether the volatile compound of Leprince is the same as that referred to by Chevalier.

We made several attempts to secure samples of the European mistletoe, and finally one of the Eastern drug firms sent us a fluid extract which they stated was made from *Viscum album*. This fluid extract caused a rise in blood pressure like that produced from *Phoradendron flavescens*, and we suspected that the firm was confusing the genus and using the American plant. Ostenberg[7] found *p*-hydroxyphenylethylamine in this fluid extract, and, trusting to the firm's statement, reported that *Viscum album* contained this compound.

Since the appearance of Ostenberg's paper, we have obtained *Viscum album* from Italy and also from England.[8] Fresh 30 per cent alcoholic extracts of both lots caused a fall in blood pressure in dogs with cut vagi, and from these extracts we obtained no *p*-hydroxyphenylethylamine by the methods described in our previous papers. The ether extract of the alkaline solution yielded an oxalate, but the intravenous injection of 15 mg. did not cause a rise in blood pressure.

A specimen of *Phoradendron villosum*, which was parasitic to *Quercus lobata*, was obtained from the Stanford campus. This sample contained leaves and twigs. A 30 per cent alcoholic extract of this plant produced a rise in blood pressure and we obtained an oxalate, by our method, which raised the blood pressure and accelerated the cardiac rate in dogs. From the oxalate a dibenzoyl compound was made which melted at 170°C., corresponding to the melting point of the dibenzoyl compound of *p*-hydroxyphenylethylamine.

Phoradendron villosum parasitic to an oak (variety not given) was obtained near Redwood City, California. A 30 per cent alcoholic extract of this produced a rise in blood pressure and yielded an active oxalate.

Arceuthobium occidentale (Jepson), parasitic to a Monterey pine (*Pinus radiata*), was obtained from near Pacific Grove, California. An alcohol extract (1 cc. = 1 gm. of the fresh plant) produced no rise in blood pressure. This plant yielded no oxalate.[9]

[7] Ostenberg, Z., *Proc. Soc. Exper. Biol. and Med.*, 1915, xii, 174.

[8] The plants from England were given us by Parke, Davis and Co., and the identification was confirmed through the courtesy of Dr. W. W. Stockberger of the U. S. Dept. of Agriculture. Those from Italy were obtained through Professor R. Dohrn of Naples, Italy.

[9] This plant was obtained through the kindness of Professor George Peirce.

Professor Thornber, of the University of Arizona, sent us samples of *Phoradendron flavescens*, variety *macrophylla* and *Phoradendron californicum*. Intravenous injections of an alcohol-aqueous extract of both plants caused a rise in blood pressure similar to that produced by *Phoradendron flavescens* and from both were obtained a dibenzoyl compound, a picrate, and an oxalate, which had the same melting points as those made from the base we obtained from *Phoradendron flavescens*. Extracts of a sample of *Phoradendron villosum* obtained from Professor Thornber produced no rise in blood pressure and yielded no *p*-hydroxyphenylethylamine, although it yielded an inactive oxalate from an ethereal shaking of an alkaline extract. This sample contained no leaves.[10] Whether this difference in the results obtained from the Arizona and the California varieties is due to the fact that in one case the sample contained leaves and in the other none, has not been investigated.

30 per cent alcoholic extracts of *Phoradendron bolleanum*,[11] *Phoradendron juniperinum*, and *Razoumofskya cryptopoda* (Coville) in which 1 cc. was supposed to represent 1 gm. of the dried plants, were injected intravenously into dogs. It was found that the extract of *Phoradendron bolleanum* (1.5 cc.), in one experiment, produced a slight rise in blood pressure, but in another experiment 1 cc. produced merely a fall. From this plant a small amount of an oxalate was obtained, but not sufficient to make a blood pressure test. It reacted with Moerner's test like *p*-hydroxyphenylethylamine. The extract of *Razoumofskya cryptopoda* and of *Phoradendron juniperinum* produced a fall in blood pressure, but no rise. Neither yielded an oxalate.

In 1911 one of us[12] reported that *Phoradendron juniperinum*, which was obtained from Arizona, seemed to be active, while *Phoradendron villosum*, obtained from the same state, seemed to be inactive. Unfortunately our

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[10] Extracts of both the pituitary and of the suprarenal glands yield an inactive oxalate from an ethereal shaking of an alkaline extract.

[11] These were received from Professor Thornber. The *Phoradendron bolleanum* was parasitic to the white fir, *Abies concolor;* the *Phoradendron juniperinum* grew on *Juniperus monosperna* and was obtained at an altitude of about 5,000 feet. *Razoumofskya cryptopoda* was parasitic to the Arizona pine, *Pinus arizonica*, in the high mountains of Arizona.

[12] Crawford, A. C., *Jour. Am. Med. Assn.*, 1911, lvii, 865.

original tracings are not now available. In this connection it may be remembered that the Indians of Mendocino County, California, believe that only the mistletoes grown on certain trees are poisonous.[13]

Livon[14] has reported that the mistletoe which he calls *Arceuthobium juniperorum*, Reynier, or *Razoumofskya caucasica* Hoffmann, exerts both a hypertensor and a hypotensor action. The first action he attributes to calcium malate, while the latter action he believes to be due to the same hypotensor compound which occurs in *Viscum album*.

The question arises whether *p*-hydroxyphenylethylamine also caused the increased urinary secretion observed in dogs as a result of the intravenous injection of extracts of *Phoradendron flavescens*. We injected the commercial preparation (tyramine) into the veins of narcotized dogs with cannulas in the ureters, and found that the urinary secretion first diminished, then increased. The intravenous injection of tyramine first diminished, then increased the renal volume (dog). But *p*-hydroxyphenylethylamine may not be the only substance responsible for the increase in urinary secretion, as *Viscum album* is claimed to have a diuretic action, yet contains no *p*-hydroxyphenylethylamine. How far the action of *p*-hydroxyphenylethylamine is modified by the presence of colloids, etc., in extracts of mistletoe remains to be seen.

[13] Chesnut, V. K., *Contr. U. S. Nat. Herbarium*, 1900–02, vii, 344.

[14] Livon, C., *Compt. rend. Soc. de biol.*, 1912, lxxii, 1111; 1912, lxxiii, 363. Gerber, C., and Cotte, J., *ibid.*, 1908, lxiv, 781, 1180.

CONTENTS.

THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER
MEMORIAL FUND

THE CASEIN OF GOAT'S MILK.

By ALFRED W. BOSWORTH and LUCIUS L. VAN SLYKE.

(From the Chemical Laboratory of the New York State Agricultural Experiment Station, Geneva.)

(Received for publication, January 8, 1916.)

In studying the composition of goat's milk in this laboratory, we found it necessary to know the percentage of phosphorus in the casein prepared from such milk and also the power of this casein to combine with bases to form caseinates. We have prepared casein from goat's milk by the method used in our study of the casein of cow's milk.[1] However, we found it even more difficult to remove all of the calcium phosphate, owing to the fact, shown in the following article, that goat's milk contains both tri- and di-calcium phosphate; but, by repeating the process of precipitation and redissolving in the presence of neutral potassium oxalate, we finally obtained a preparation in which the percentage of ash was about 0.36.

Below is given the analysis of a preparation of casein made from goat's milk together with the analysis of casein of cow's milk.

Percentage of Constituents in Moisture-Free and Ash-Free Casein.

	From goat's milk.	From cow's milk.
Ash...............................	0.36	0.06
Carbon...........................	52.50	53.50
Hydrogen.........................	7.16	7.13
Nitrogen.........................	15.67	15.80
Phosphorus.......................	0.71	0.71
Sulfur...........................	0.71	0.72
Oxygen (by difference)...........	23.25	22.08

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 203.

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Relation of Casein of Goat's Milk to Bases.

By applying the details of the methods already published by us[2] we find that the casein of goat's milk combines with bases to form the same series of salts (caseinates) as in the case of the casein of cow's milk. The results of our work are briefly embodied in the following summary.

(1) 1 gm. of casein combines with 9×10^{-4} gm. equivalents of base to form a compound (caseinate) neutral to phenolphthalein. (2) 1 gm. of casein combines with 1.125×10^{-4} gm. equivalents of ammonia, sodium, or potassium to form a soluble caseinate; there is no other combination of casein with a base containing less than 1.125×10^{-4} gm. equivalents of base for 1 gm. of casein. (3) 1 gm. of casein combines with 2.25×10^{-4} gm. equivalents of calcium to form a caseinate soluble in water. This caseinate is insoluble in water in the presence of more than a trace of any soluble salt of calcium, barium, or strontium. (4) 1 gm. of casein combines with 1.125×10^{-4} gm. equivalents of calcium to form a caseinate insoluble in water. This compound is soluble in a warm 5 per cent solution of sodium chloride, the solvent action being due to an interchange of bases by double decomposition, with the consequent production of soluble sodium caseinate.[3]

Valency and Molecular Weight of Casein of Goat's Milk.

From the preceding data, it can be seen that the valency of casein from goat's milk, like that of casein from cow's milk, is 8.

$$\frac{9 \times 10^{-4}}{1.125 \times 10^{-4}} = 8$$

The molecular weight would, therefore, be $\dfrac{1}{1.125 \times 10^{-4}} = 8{,}888$, and the equivalent weight would be $\dfrac{8{,}888}{8} = 1{,}111$.

On the basis of the sulfur content we would have:

$$\text{Molecular weight} = N \left(\frac{32.07}{0.71}\right) \times 100 = N\ (4{,}393).$$

[2] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 211.
[3] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1913, xiv, 217.

If N equals 2, the molecular weight equals 8,786.

On the basis of the amount of phosphorus contained in casein, we have:

$$\text{Molecular weight} = N\left(\frac{31.04}{0.71}\right) \times 100 = N\ (4,372).$$

If N equals 2, the molecular weight equals 8,744.

SUMMARY.

On the basis of the analytical results obtained in the study of the compounds formed by casein of goat's milk with bases, taken together with the amount of phosphorus and sulfur found in such casein, the molecular weight is 8,888, and the valency of the protein molecule in basic caseinates is 8.

THE SOLUBLE AND INSOLUBLE COMPOUNDS OF GOAT'S MILK.

By ALFRED W. BOSWORTH AND LUCIUS L. VAN SLYKE.

(*From the Chemical Laboratory of the New York State Agricultural Experiment Station, Geneva.*)

When milk is brought into contact with a porous earthenware filter, the serum, consisting of the water and the compounds in true solution, passes through the filter, while the compounds insoluble in water, which are present in suspension (fat and calcium phosphate), or in the form of colloids (casein), remain on the surface of the filter. The details of the method used in separating milk-serum from the other constituents of milk have been fully given elsewhere.[1]

By determining the amounts of the constituents present in the whole milk and in the serum, we are enabled to determine by difference the quantities present in the milk in suspension or colloidal solution.

Table I gives the figures obtained by us in working with four samples of goat's milk, in which only the more prominent constituents were determined.

A study of the data contained in Table I enables us to show the general relation of the constituents of milk to the constituents of milk-serum. The following form of statement furnishes a clear summary of the facts.

1. Milk constituents in true solution in milk.	2. Milk constituents partly in solution and partly in suspension or colloidal solution.	3. Milk constituents entirely in suspension or colloidal solution.
Sugar.	Albumin.	Fat.
Potassium.	Inorganic phosphates.	Casein.
Sodium.	Calcium.	
Chlorine.	Magnesium.	
	Citrates.	

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 136.

TABLE I.

Constituents of Goat's Milk.

Constituents.	Sample 1.			Sample 2.			Sample 3.			Sample 4.		
	In original milk, per 100 cc.	In milk-serum, per 100 cc.	Milk constituents in serum.	In original milk, per 100 cc.	In milk-serum, per 100 cc.	Milk constituents in serum.	In original milk, per 100 cc.	In milk-serum, per 100 cc.	Milk constituents in serum.	In original milk, per 100 cc.	In milk-serum, per 100 cc.	Milk constituents in serum.
	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.	per cent	gm.	gm.	per cent
Fat	3.80	0.00	0.00	2.20	0.00	0.00	3.70	0.00	0.00	3.00	0.00	0.00
Sugar	5.22	5.22	100.00	4.82	4.82	100.00	4.52	4.52	100.00	4.45	4.45	100.00
Nitrogen, total	0.520	0.114	21.92	0.492	0.104	21.13	0.450	0.078	17.33	0.404	0.075	18.56
" in casein	0.340	0.000	00.00	0.353	0.000	00.00	0.316	0.000	0.00	0.267	0.000	00.00
" " albumin	0.090	0.035	38.89	0.059	0.024	40.67	0.060	0.012	20.00	0.067	0.010	14.92
" " other proteins	0.090	0.079	87.78	0.080	0.080	100.00	0.074	0.066	89.19	0.070	0.065	92.86
Citric acid	0.1307	0.0991	75.82	0.1485	0.1322	89.02	0.1712	0.1283	74.89	0.1531	0.1379	90.07
Phosphorus, total	0.1206	0.0502	41.63	0.0906	0.0390	43.05	0.0862	0.0392	54.48	0.0841	0.0413	49.11
" organic	0.0153	0.000	00.00	0.0159	0.000	00.00	0.0142	0.0000	00.00	0.0120	0.000	00.00
" inorganic	0.1053	0.0502	47.67	0.0747	0.0390	52.21	0.0720	0.0392	54.44	0.0721	0.0413	57.28
Calcium	0.1632	0.0414	25.37	0.1500	0.0534	35.60	0.1240	0.0351	28.31	0.1144	0.0374	32.69
Magnesium	0.0204	0.0128	62.75	0.0212	0.0132	62.26	0.0206	0.0128	62.19	0.0236	0.0144	61.02
Potassium	0.1700	0.1724	100.00	0.1656	0.1784	100.00	0.2284	0.2284	100.00	0.2084	0.2084	100.00
Sodium	0.0380	0.0380	100.00	0.0400	0.0396	100.00	0.0352	0.0352	100.00	0.0372	0.0376	100.00
Chlorine	0.1578	0.1581	100.00	0.1811	0.1839	100.00	0.2171	0.2168	100.00	0.2597	0.2665	100.00

Properties and Composition of Portion of Milk in Suspension or Colloidal Solution.

Some of the constituents of milk are suspended in the form of solid particles in such an extremely fine state of division that they pass through the pores of filter-paper, and do not settle as a sediment on standing, but remain permanently afloat. They cannot be seen except by ultramicroscopic methods. When substances are in such a condition, they are said to form a colloidal solution. In passing milk through the Pasteur-Chamberland filtering-tube, the constituents in suspension as solid particles, and in colloidal solution, are retained in a solid mass on the outside of the tube and can therefore be readily obtained for study.

Appearance.—When prepared by the method of filtration previously described, the insoluble portion of fat-free milk collecting on the outside of the filtering-tube is grayish to greenish

white in color, of a glistening appearance, and of gelatinous consistency. When dried without purification by treatment with alcohol, etc., it resembles dried white of egg.

Behavior with Water.—The deposit of insoluble milk constituents on the outside of the filtering-tube, when removed and shaken vigorously in a flask with distilled water, goes into suspension, and the mixture has the opaque, white appearance of the original milk. The deposit is, of course, more or less mixed with adhering soluble constituents, but can be readily purified by shaking with distilled water and filtering several times. The purified material goes readily into suspension on shaking with water, and, if treated with a preservative, will remain indefinitely without change other than the separation of fat-globules. It has been held by some that the citrates of milk perform the function of holding the insoluble phosphates in suspension, but this belief is not supported by the behavior of the insoluble portion, as shown in our experiments.

Reaction.—A suspension of the insoluble constituents of milk, prepared in the manner described above, is alkaline to phenolphthalein. We purified the deposit obtained from 1,000 cc. of milk and made a suspension of it in water; it was found to be neutral to phenolphthalein, but, after the addition of 10 cc. of neutral potassium oxalate, it developed a strongly alkaline reaction to phenolphthalein. We interpret this behavior to mean that the insoluble portion of goat's milk contains an alkaline phosphate, which, being insoluble, does not affect the indicator; but the addition of a solution of neutral potassium oxalate decomposes the insoluble phosphate, with the production of soluble tri-potassium phosphate, which reacts alkaline to phenolphthalein, as will be shown later (page 181).

Composition.—As shown in Table I, the insoluble portion of goat's milk contains as chief constituents, fat, protein, inorganic phosphates, calcium, and magnesium. The fat can be separated from the other constituents by extraction with ether or by removal from the original milk by means of centrifugal force. We have previously shown that albumin may be adsorbed to some extent by casein[2] and thus partly retained on the filter. In view

[2] Van Slyke and Bosworth, *J. Biol. Chem.*, 1915, xx, 138.

of these conditions, our studies have been limited to the acids (casein and phosphates) and the bases (calcium and magnesium) present in this portion of the milk.

Relation between Acids and Bases in the Insoluble Portion of Goat's Milk.—In order to bring out clearly the relation existing between the bases and the acids in the insoluble portion of the milk, we have prepared Tables II and III. Table II is obtained from the figures in Table I, and Table III is obtained by converting the percentage figures of Table II into gm. equivalents per

TABLE II.

Amounts of Casein, Inorganic Phosphorus, Citric Acid, Calcium, and Magnesium in the Insoluble Portion of Goat's Milk.

Sample No.	Casein.	Inorganic phosphorus.	Citric acid.	Calcium.	Magnesium.
	per cent	per cent	per cent	per cent	per cent
1	2.17	0.0551	0.0316	0.1218	0.0076
2	2.25	0.0357	0.0163	0.0966	0.0080
3	2.01	0.0328	0.0429	0.0889	0.0078
4	1.70	0.0308	0.0152	0.0770	0.0092

TABLE III.

Amounts of Acids and Bases in Table II Expressed as Gm. Equivalents Per 100 Cc. of Milk.

Sample No.	Casein as octavalent acid.	Phosphorus as di-basic acid.	Citric acid as tri-basic acid.	Sum of values of acids.	Calcium.	Magnesium.	Sum of values of bases.	Excess of base.
1	19.5×10^{-4}	35.5×10^{-4}	4.9×10^{-4}	59.9×10^{-4}	60.9×10^{-4}	6.3×10^{-4}	67.2×10^{-4}	7.3×10^{-4}
2	20.3×10^{-4}	23.0×10^{-4}	2.5×10^{-4}	45.8×10^{-4}	48.3×10^{-4}	6.7×10^{-4}	55.0×10^{-4}	9.2×10^{-4}
3	18.1×10^{-4}	21.2×10^{-4}	6.7×10^{-4}	46.0×10^{-4}	44.5×10^{-4}	6.5×10^{-4}	51.0×10^{-4}	5.0×10^{-4}
4	15.3×10^{-4}	19.9×10^{-4}	2.4×10^{-4}	37.6×10^{-4}	37.2×10^{-4}	7.7×10^{-4}	44.9×10^{-4}	7.3×10^{-4}

100 cc. of milk. In making these calculations, we have considered the casein of goat's milk to be octavalent, 1 gm. combining with 9×10^{-1} gm. equivalents of base to form a compound neutral to phenolphthalein (page 174). The figures for organic phosphorus are obtained by subtracting the amount of phosphorus in the casein (0.71 per cent) from the total phosphorus.

The last column of Table III shows that the amounts of bases in the insoluble portion of goat's milk are in excess of the amounts required to form with the acids that are present salts neutral to

phenolphthalein. There is reason to believe that this excess of base is present in combination with the phosphorus in the form of tri-calcium phosphate. The evidence supporting this belief is furnished by an examination of the insoluble portion of goat's milk obtained by separation through centrifugal force in a cream-separator.[3]

The bowl of a cream-separator was filled with about 1,000 cc. of fat-free milk and was whirled for 2 hours at a speed of 5,000 revolutions a minute. The liquid portion of the milk was then

TABLE IV.

Composition of Insoluble Portion or "Separator Slime" of Goat's Milk.

Deposit formed by whirling two 2 hour periods.	Casein.	Total phosphorus.	Phosphorus in casein.	Phosphorus as phosphate.	Calcium.	Ratio of organic to insoluble inorganic phosphorus.
	per cent	per cent	per ce it	per cent	per cent	
1st 2 hrs.	75.56	2.57	0.54	2.03	4.46	1: 3.72
2nd 2 hrs.	73.17	2.59	0.53	2.06	4.40	1: 3.81

TABLE V.

Amounts of Acids and Bases in Table IV Expressed as Gm. Equivalents per 100 Cc. of Milk.

	Casein as gm. equivalents of acid.	Phosphate as gm. equivalents of di-basic acid.	Sum of gm. equivalents of casein and phosphates.	Gm. equivalents of calcium.	Excess of calcium as gm. equivalents.
In 1st deposit	68 $\times 10^{-3}$	132$\times 10^{-3}$	200 $\times 10^{-3}$	226$\times 10^{-3}$	26 $\times 10^{-3}$
" 2nd "	65.9$\times 10^{-3}$	132$\times 10^{-3}$	198.7$\times 10^{-3}$	220$\times 10^{-3}$	21.3$\times 10^{-3}$

taken from the bowl and the deposit ("separator slime"), which had collected on the walls of the separator-bowl, was removed, treated with alcohol and ether, and dried in the manner described.[4] This liquid portion of the milk that had been whirled for 2 hours was again placed in the separator-bowl and whirled for an additional 2 hours, when the deposit was again removed and treated as before. After the removal of the second deposit, the liquid portion of the milk remaining in the separator was nearly as

[3] Van Slyke and Bosworth, J. Biol. Chem., 1915, xx, 144.
[4] Van Slyke and Bosworth, J. Biol. Chem., 1915, xx, 145.

clear as whey, since most of the suspended phosphates and casein had been deposited on the walls of the bowl during the whirling.

The results of the analysis of the dried deposit obtained after each 2 hour period of whirling are given in Table IV.

Expressing the data in Table IV in the form of gm. equivalents per 100 cc. of milk, we obtain the figures contained in Table V.

These figures show an amount of calcium in excess of that required to form salts neutral to phenolphthalein with the acids present, casein and phosphoric acid. It should be stated here that these deposits of "separator slime" contain no citric acid. When the deposit is suspended in water in the presence of phenolphthalein, no color appears; but, if 1 cc. of a neutral solution of potassium oxalate is added, a pink color appears, becoming more intense after standing for 1 or 2 minutes. The most obvious explanation of this behavior is that the addition of neutral potassium oxalate results in a reaction between it and the tri-calcium phosphate, tri-potassium phosphate being formed and turning · the indicator pink.

The Acidity of Goat's Milk and Its Serum.

When the determination of the acidity of goat's milk and its serum is made by adding neutral potassium oxalate and filtering previous to titration, according to the method recently published by us,[5] results are obtained as given in Table VI.

There is observable a marked difference between the acidity of the whole milk and that of its serum. This condition is accounted for by the following explanation. In normal goat's milk, which is nearly neutral, there is present in suspension dicalcium phosphate; this phosphate, in the presence of the water in the milk, undergoes more or less decomposition, for, according to the work of Cameron and Hurst,[6] water in contact with a solid phase containing calcium phosphate dissolves more acid than base from the solid phase. The result of this action is that the insoluble portion of the milk becomes more basic than the whole milk, as shown above, while the soluble portion becomes more

[5] Van Slyke and Bosworth, *J. Biol. Chem.*, 1914, xix, 73.

[6] Cameron, F. K., and Hurst, L. A., *J. Am. Chem. Soc.*, 1904, xxvi, 905.

TABLE VI.

Acidity of Goat's Milk and Its Serum.

Sample No.	0.1 N alkali required to neutralise 100 cc. of	
	Milk.	Serum.
	cc.	cc.
1	1.4	6.6
2	3.0	5.6
3	0.6	4.8
4	0.3	5.8
5	0.0	5.0
6	0.0	3.0

acid. When the solid and liquid portions of milk are separated by treatment on a porous porcelain filter, the soluble acid passes into the serum, the basic compound remaining in the insoluble portion.

In determining the acidity of milk, it should be remembered that the presence of di-calcium phosphate furnishes conditions favorable to the formation of acid phosphates and tri-calcium phosphate, as already shown,[5] resulting in an increased requirement of alkali for neutralization and apparently a higher acidity if the milk is titrated directly. For this reason the lower acidity obtained after the removal of calcium by neutral potassium oxalate is believed to represent the real acidity of the milk. This point is well illustrated by the following figures.

TABLE VII.

Sample No.	0.1 N alkali required to neutralize 100 cc. of milk.	
	Before addition of neutral potassium oxalate.	After addition of neutral potassium oxalate.
	cc.	cc.
1	17.6	1.5
2	21.0	3.0
3	26.0	3.2
4	25.6	8.0
5	20.6	1.4

Compounds in Goat's Milk.

Based on the result of our study of the composition of goat's milk, the following arrangement is tentatively suggested as representing the forms in which the constituents may be present:

		per cent
Fat		3.80
Proteins, combined with calcium		3.10
Milk-sugar		4.50

Salts		per cent	
Di-calcium	phosphate	0.092	
Tri-calcium	"	0.062	
Di-magnesium	"	0.068	
Tri-magnesium	"	0.024	
Mono-potassium	"	0.073	0.94
Potassium citrate		0.250	
Potassium chloride		0.160	
Calcium	"	0.115	
Sodium	"	0.095	
Total solids			12.34

SUMMARY.

1. Goat's milk contains two general classes of compounds, those in true solution and those in suspension or colloidal solution. These two portions can be separated for study by filtering milk through a porous earthenware filter like the Pasteur-Chamberland filtering-tube.

2. Serum prepared from fresh milk of goats is nearly transparent, with a faint greenish yellow tinge and slight opalescence. The following constituents are in true solution: sugar, potassium, sodium, chlorine. The following are partly in solution and partly in suspension or colloidal solution: albumin, inorganic phosphates, calcium, magnesium, citrates. The following are entirely in suspension or colloidal solution: fat, casein.

3. The insoluble portion of milk, freshly prepared and moist, is grayish to greenish white in color, of a glistening appearance, and of gelatinous consistency. When shaken with water, it goes into suspension, forming a mixture having the opaque, white appearance of milk. Such a suspension is neutral to phenolphthalein. When purified, the insoluble portion consists of neu-

tral calcium caseinate (casein Ca$_4$), di- and tri-calcium, and magnesium phosphates.

4. The insoluble portion, suspended in water and treated with neutral potassium oxalate, reacts alkaline to phenolphthalein, indicating the presence of tri-calcium phosphate. This is shown also by a quantitative comparison of the amount of bases and acids.

5. The acidity of the serum is considerably greater than that shown by the milk after treatment with neutral calcium oxalate. This is accounted for by the presence of di-calcium phosphate.

6. The data presented furnish a basis for suggesting the forms of compounds in which the salts may be present.

A COMPARISON OF THE COMPOSITION OF COW'S MILK, GOAT'S MILK, AND HUMAN MILK.

By ALFRED W. BOSWORTH and LUCIUS L. VAN SLYKE.

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We have published statements attempting to indicate the individual forms or compounds in which the salts exist in cow's milk and in human milk.[1] The same has been done for goat's milk in the preceding paper. It is a matter of interest to bring these results together in order to see in what respects the three kinds of milk differ. It is understood, of course, that the arrange-

Compounds in Cow's Milk, Goat's Milk, and Human Milk.

Compounds.	Cow's milk.	Goat's milk.	Human milk.
	per cent	*per cent*	*per cent*
Fat	3.90	3.80	3.30
Milk-sugar	4.90	4.50	6.50
Proteins, combined with calcium	3.20	3.10	1.50
Salts	0.901	0.939	0.313
Di-calcium phosphate	0.175	0.092	0.000
Tri-calcium "	0.000	0.062	0.000
Mono-magnesium phosphate	0.103	0.000	0.027
Di-magnesium "	0.000	0.068	0.000
Tri-magnesium "	0.000	0.024	0.000
Mono-potassium "	0.000	0.073	0.069
Di-potassium "	0.230	0.000	0.000
Potassium citrate	0.052	0.250	0.103
Sodium "	0.222	0.000	0.055
Potassium chloride	0.000	0.160	0.000
Sodium "	0.000	0.095	0.000
Calcium "	0.119	0.115	0.059

[1] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 151. Bosworth, A. W., *ibid.*, 1915, xx, 707.

ment of the constituents is based upon a limited amount of work. We know that there is variation in the quantitative relations of the different compounds. There remains much work to be done in applying our methods to the study of milks produced under a variety of known conditions. The present results are, therefore, tentative in character and are likely to be modified by more extensive work.

In the preceding table the figures which have a special interest are those relating to the salts, and we notice the following points in relation to these compounds.

Phosphates.

Cow's Milk.—The insoluble phosphate is di-calcium phosphate; tri-calcium, di- and tri-magnesium phosphates do not appear to be present. The soluble phosphates are mono-magnesium and di-potassium, which constitute about two-thirds of the total phosphates.

Goat's Milk.—This differs from cow's milk (1) in containing tri-calcium, di- and tri-magnesium, and mono-potassium phosphates, and (2) in containing no mono-magnesium or di-potassium phosphates.

Human Milk.—This differs noticeably from both cow's milk and goat's milk in containing no insoluble phosphates, but only the soluble compounds, mono-magnesium and mono-potassium phosphates. The phosphates in human milk are much less in amount than in cow's or goat's milk.

Citrates.

All three milks contain potassium citrate, while cow's milk and human milk contain sodium citrate also.

Chlorides.

Chlorides are present in goat's milk in much larger amounts than in cow's milk or human milk; the amount in cow's milk is considerably larger than in human milk. In cow's milk and human milk the chloride appears to be calcium chloride, while in goat's milk potassium and sodium chlorides are also present.

Total Salts.

The total amount of salts in human milk is about one-third that in cow's milk or goat's milk. The number of different salts appears to be greatest in goat's milk and least in human milk.

We have carried out numerous experiments in studying the changes that take place in milk as a result of souring and the results have been uniform in agreement. We shall, therefore, instead of presenting all of our data, give such as will fully illustrate the ascertained facts. We will give first the analytical results obtained with milk at the end of 60 hours and then a set of results obtained at frequent intervals during a period of 96 hours.

Changes in Milk in 60 Hours Caused by Souring.

Before considering the detailed changes that are produced in the constituents of milk during the various stages of progressive souring, we will show the final changes that take place when the souring process has been practically completed, which under ordinary conditions should be in about 60 hours.

4 liters of fresh milk, divided into two portions, of 2 liters each, were treated as follows: To one portion chloroform was added at once and the mixture was passed through a porous porcelain filter. The second portion was allowed to sour at room temperature after inoculation with a culture containing *Bacterium lactis acidi* and *Bacterium lactis aerogenes*. At the end of 60 hours, this was filtered through a porous porcelain filter. Analysis was made of the fresh milk, of the serum of the fresh milk, and of the serum of the sour milk, the results of which are given in Table I.

A study of the results in Table I shows in general that the main effects produced by the souring of milk are the conversion of more or less milk-sugar into lactic acid, which causes precipitation of casein and makes soluble the other insoluble constituents. Stated in more detail, we notice the following results:

1. The amount of sugar in milk is decreased through the action of the lactic acid bacteria. In this case there is a loss of 1.27 gm. for 5.75 gm. of sugar per 100 cc. of milk, or 22 per cent. The amount of lactic acid formed is 1.124 gm., or 88.5 per cent of the sugar decomposed is converted into lactic acid.

2. The citric acid of milk completely disappears, being decomposed into acetic acid and carbon dioxide by the action of *Bacterium lactis aerogenes*.

3. Those inorganic constituents of normal milk that are insoluble are made soluble by the acid resulting from bacterial action.

4. The albumin, part of which in normal milk fails to pass through a porous porcelain filter, is so changed in sour milk as to pass completely through such a filter.

5. Calcium caseinate of normal milk is completely converted into the free protein, uncombined with any base, which is precipitated, and calcium lactate is formed, soluble in the serum.

TABLE I.

Changes in the Condition of Milk Constituents as a Result of Souring.

Constituents.	Original milk, 100 cc.	Serum from fresh milk.		Serum from sour milk.	
		Serum, 100 cc.	Percentage of milk constituents in serum.	Serum, 100 cc.	Percentage of milk constituents in serum.
	gm.	*gm.*		*gm.*	
Sugar....................	5.75	5.75	100.00	4.48	—
Casein..................	3.07	0.00	0.00	0.00	0.00
Albumin................	0.506	0.188	37.15	0.506	100.00
Nitrogen in other compounds...............	0.049	0.049	100.00	0.049	100.00
Citric acid.............	0.237	0.237	100.00	0.000	0.00*
Phosphorus, inorganic...	0.087	0.056	64.40	0.090	100.00
Calcium.................	0.144	0.048	33.33	0.148	100.00
Magnesium..............	0.013	0.007	53.85	0.014	100.00
Potassium..............	0.120	0.124	100.00	0.120	100.00
Sodium.................	0.055	0.057	100.00	0.058	100.00
Chlorine...............	0.076	0.081	100.00	0.079	100.00
Ash....................	0.725	0.400	55.17	0.690	95.17**
Lactic acid.............	0.000	0.000	0.00	1.124	—†

* During the process of souring the citric acid is completely changed by the action of *Bacterium lactis aerogenes* into acetic acid and carbon dioxide, as shown by Bosworth, A. W., and Prucha, M. J., *J. Biol. Chem.*, 1910, viii, 479.

** In determining the total ash of milk, the organic phosphorus of the casein is included in the ash as phosphoric acid, but in the serum this organic phosphorus is not present and therefore the amount of ash in serum from sour milk is less than that in fresh milk.

† Some of the lactic acid is adsorbed by the casein and therefore not all of it is found in the serum (Van Slyke, L. L., and Van Slyke, D. D., *Am. Chem. J.*, 1907, xxxviii, 383).

*Changes in Milk at Different Stages of Souring.*

In addition to the ultimate changes, it is desirable to know something of the rate of chemical change taking place when milk sours. We have carried on numerous sets of experiments, but present here the results of only one, which is typical of all under the same conditions.

The milk used in this experiment was fresh morning milk, which had been pasteurized and run through a separator to remove the fat. The milk was inoculated with a pure culture of *Bacterium lactis acidi* and kept at a temperature of about 32.2°C. (90°F.), samples being taken for analysis at intervals and chloroformed at once in order to prevent further bacterial action. Sampling began after the milk had stood 4 hours and continued at intervals of an hour or less for 16 hours; after 24 hours samples were taken every 24 hours up to the end of 96 hours.

The constituents to which attention was given are the following: (1) sugar, (2) lactic acid, (3) acidity before and after passing through porcelain filter, (4) nitrogen in serum, (5) nitrogen as albumin in serum, (6) soluble and insoluble inorganic phosphorus, (7) soluble and insoluble calcium and magnesium, (8) calcium as di-calcium phosphate, (9) calcium derived from calcium caseinate.

The methods by which the amounts of total, soluble, and insoluble calcium, magnesium, and phosphorus were obtained have been fully described in our previous work.[1]

Considerable time was devoted to attempts to determine separately the amounts of free lactic acid and combined lactic acid, but no satisfactory method could be devised for making such distinction in the presence of acid phosphates. This is due to the conditions existing in sour milk, where we have present acid calcium phosphate and calcium lactate, and, in advanced stages of souring, some free lactic acid. Extraction of a solution containing lactates in the presence of acid phosphates results in the following reaction, which gives an apparent quantity of free lactic acid much greater than the actual amount:

$$CaH_4P_2O_8 + Ca(C_3H_5O_3)_2 = 2CaHPO_4 + 2HC_3H_5O_3$$
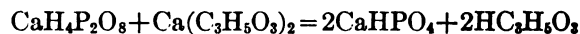
In a solution containing free lactic acid, lactates, and phosphates, the lactate and phosphate undergo mutual reaction, forming free

lactic acid and di-calcium phosphate. While we find it possible to extract with ether all the free lactic acid from a solution containing these compounds, we are unable to tell with accuracy at what point the conversion of lactate into free lactic acid begins. It is possible by extraction to remove the lactic acid of the lactate completely from a solution containing calcium lactate and acid calcium phosphate provided the phosphate is present in excess, but we have thus far found no satisfactory way of making a separate determination of free lactic acid in the presence of these salts by extraction. We hope, however, to do this later by determination of the free hydrogen ions.

TABLE II.

Composition of Fresh Milk before Souring.

	In 100 cc.		In 100 cc.
	gm.		*gm.*
Nitrogen, total	0.5964	Phosphorus, inorganic, insoluble	0.0399
" as casein	0.4704		
" " albumin	0.0762	Calcium, total	0.1571
" other than casein		" soluble	0.0427
and albumin	0.0498	" insoluble	0.1144
Nitrogen in serum	0.0770	" " as $CaHPO_4$	0.0515
Phosphorus, total	0.1039	" combined with protein	0.0629
" in casein	0.0211		
" inorganic	0.0828	Magnesium, total	0.0158
" " soluble	0.0429	" soluble	0.0112
		" insoluble	0.0046

Before giving the results of the work for the sour milk in its various progressive stages of change, we give in Table II a statement of the compounds present in the fresh milk previous to souring, as a convenience for reference in interpreting the data in Table III.

Conversion of Milk-Sugar into Lactic Acid in the Souring of Milk.

Table III gives (1) the amount of sugar in the milk when fresh and at intervals up to the end of 96 hours, and (2) the amount of lactic acid found. The lactic acid was determined by extraction with ether and conversion into zinc lactate.

The fermentation of the milk-sugar, under the conditions of the experiment, is slow during the initial period but between the 10th and 25th hours most of the change takes place. With increase of lactic acid beyond 0.7 per cent, bacterial activity is much reduced. Between the 48th and 96th hours only a slight change in sugar takes place. Attention is called to the apparent discrepancy at 48 and 96 hours in the relation of the amount of lactic acid formed to the amount of sugar decomposed. Apparently more acid is formed than can be accounted for by the amount of sugar fermented. This is due to the fact that the acetic acid produced by the fermentation of the citric acid is extracted by ether and weighed with the lactic acid.

TABLE III.

Formation of Lactic Acid in the Souring of Milk.

Age of milk when sampled.	100 cc. of milk.			Milk-sugar changed.	Fermented sugar changed into lactic acid.
	Sugar.	Amount of sugar changed.	Lactic acid.		
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
Fresh.	5.30	—	0.000	—	—
10	5.07	0.23	0.200	4.3	87
12	4.83	0.47	0.330	8.9	70
14	4.68	0.62	0.513	11.7	82
19	4.58	0.72	0.671	13.6	93
25	4.42	0.88	0.665	16.6	75.5
48	4.30	1.00	1.052	18.9	—
96	4.26	1.04	1.124	20.0	—

In the last column of Table III we give the proportion of fermented milk-sugar that was changed into lactic acid. The proportions vary from 70 to 93 per cent. It is known that products other than lactic acid are formed, varying in kind and amount according to the conditions of the fermentation process.

Effect of the Souring of Milk upon Acidity of Milk and Milk-Serum.

Table IV gives (1) the amount of total acidity in the fresh milk and that found at intervals during the process of fermentation; (2) the amount of acids in the serum; (3) the amount of acids

in the portion of the milk that does not pass through the porous porcelain filter; and (4) the amount of lactic acid.

The acidity of the insoluble portion of the milk is obtained by difference. The amount or degree of acidity, in the case of the milk and milk-serum, is expressed in terms of 0.1 N alkali required to produce neutrality to phenolphthalein after removal of cal-

TABLE IV.

Acidity of Milk, Milk-Serum, etc.

Age of milk when sampled.	Acidity expressed as cc. 0.1 N alkali required to neutralise 100 cc. of milk.				Lactic acid.
	In milk, total.	In serum.	In insoluble portion.	Increase of acidity in milk.	
hrs.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Fresh.	10.0	7.8	2.2	—	—
4	12.0	8.2	3.8	2.0	1.7
5	14.0	9.6	4.4	4.0	3.8
6	15.6	10.0	5.6	5.6	5.3
7	19.6	11.8	7.8	9.6	9.2
8	21.0	13.6	7.4	11.0	10.9
9	26.0	15.6	10.4	16.0	15.7
10	33.0	17.0	16.0	23.0	22.0
11	37.0	20.6	16.4	27.0	26.7
11½	42.4	24.0	18.4	32.4	31.3
12	47.6	27.0	20.6	37.6	36.6
13	55.6*	30.2	25.4*	45.6*	45.6
13½	62.0	30.2	31.8	52.0	52.0
14	67.1	32.0	35.1	57.1	57.1
25	84.0	41.6	42.4	74.0	74.0
48	127.0	53.6	73.4	117.0	117.0
72	133.0	59.0	74.0	123.0	123.0
96	135.0	62.4	72.6	125.0	125.0

* As explained in the text above, the figures expressing total acidity in milk from 13 to 96 hours inclusive are obtained by calculation, owing to the curdling of the milk.

cium by neutral potassium oxalate. The amount of lactic acid was determined by extraction and conversion into zinc lactate.

After 12 hours the milk curdled and the acidity could not be satisfactorily determined by titration. The figures given in Table IV, Column 2, for total acidity in milk after 12 hours are obtained by adding 10 (the amount of acid in the fresh milk) to the amount

of lactic acid found by direct determination. The figures in Column 4 under acidity of "insoluble portion" of milk and in Column 5 under "increase of acidity in milk" after 12 hours are based also upon the calculated amount of total acidity.

Attention is called to the following points connected with the results given in Table IV:

1. Increase of Total Acidity in Milk.—There is an increase of acidity in the milk; this is more or less rapid for the intervals of the first 24 hours; the increase during the second 24 hours is much less than during the first, and after 48 hours further increase is insignificant. The acidity of fresh milk is due to acid phosphates. The increase is due to the production of lactic acid resulting from the decomposition of milk-sugar. This is clearly shown by a comparison of the last two columns, in which the figures representing the increase of acidity in milk run closely parallel with those representing the amount of lactic acid obtained by direct determination.

2. Acidity of Serum.—The acidity of the serum increases during the whole observed period of 96 hours, but the increase is relatively small after the first 24 hours. The increase of acidity in the milk-serum is due to the increased formation of lactic acid in the milk. We should expect, owing to the solubility of the compounds producing acidity, to find the increase of acidity in the serum closely equal to the amount of increase of lactic acid. However, a comparison of the figures shows that the amount of acid in the serum does not increase as rapidly as lactic acid. The explanation that most readily suggests itself for this condition is that there is adsorption of the acid compounds by the particles of precipitated casein, as already stated in the third foot-note in connection with Table I.

3. Acidity of Insoluble Portion.—During the process of souring, the calcium is removed from the calcium caseinate of milk, and the resulting form of casein is able to neutralize alkali. The decalcified casein constitutes the portion of the insoluble part of milk that possesses acid properties, but, owing to its property of adsorbing acids, the insoluble part of souring milk, prepared by the method of filtration through a porous porcelain filter, shows a higher acidity than that due to the base-combining power of the decalcified casein.

Effect of the Souring of Milk upon Insoluble Calcium and Inorganic Phosphorus.

The insoluble calcium in fresh milk is in combination (1) with phosphoric acid as $CaHPO_4$ and (2) with casein as Ca_4 caseinate. With the formation of lactic acid, the di-calcium phosphate is converted into mono-calcium phosphate ($CaH_4P_2O_8$), and the calcium caseinate is changed, first, into caseinates containing less

TABLE V.

Proportions of Insoluble Calcium and Inorganic Phosphorus Dissolved in the Souring of Milk.

Age of milk when sampled.	Insoluble calcium in 100 cc. of milk.			Percentage of insoluble calcium in milk dissolved in case of		Insoluble inorganic phosphorus in 100 cc. of milk.	Percentage of insoluble inorganic phosphorus dissolved.
	Total.	In combination as $CaHPO_4$.	In combination as caseinate.	$CaHPO_4$	Caseinate.		
hrs.	*gm.*	*gm.*	*gm.*			*gm.*	
Fresh.	0.1144	0.0515	0.0629	—	—	0.0399	—
4	0.1102	0.0470	0.0632	8.7	0.0	0.0364	8.7
5	0.1090	0.0457	0.0633	11.2	0.0	0.0354	11.2
6	0.1041	0.0431	0.0610	16.3	3.2	0.0334	16.3
7	0.0974	0.0418	0.0556	18.8	11.7	0.0324	18.8
8	0.0922	0.0374	0.0548	27.4	13.0	0.0290	27.4
9	0.0775	0.0319	0.0456	38.0	27.6	0.0247	38.0
10	0.0648	0.0255	0.0393	50.5	37.6	0.0198	50.5
11	0.0560	0.0180	0.0380	65.0	39.7	0.0140	65.0
12	0.0368	0.0049	0.0319	90.5	—	0.0038	90.5
13	0.0191	0.0019	0.0172	96.3	72.7	0.0015	96.3
13½	0.0154	0.0000	0.0154	100.0	75.5	0.0000	100.0
15	0.0092	—	0.0092	—	85.4	—	—
19	0.0088	—	0.0088	—	86.0	—	—
25	0.0000	—	0.0000	—	100.0	—	—

calcium and, finally, into uncombined casein. Table V gives for different intervals of the process of souring the following determinations: (1) insoluble calcium, total; (2) calcium in combination as $CaHPO_4$; (3) calcium in calcium caseinate; (4) amount of calcium as $CaHPO_4$ that is dissolved; (5) amount of calcium in calcium caseinate that is dissolved; (6) insoluble inorganic phosphorus; and (7) inorganic insoluble phosphorus dissolved. The values given for fresh milk are obtained from Table II.

In studying the data in Table V, we call attention to the following summary of the results:

1. Insoluble calcium and phosphorus in combination as $CaHPO_4$ begin to go into solution as $CaH_4P_2O_8$ and calcium lactate in a few hours and the action continues with increasing rapidity most of the time until the solution is complete at the end of $13\frac{1}{2}$ hours under the conditions of the experiments.

2. The insoluble calcium in combination with casein does not appear to be acted upon by the lactic acid as quickly as does that in $CaHPO_4$. The calcium in calcium caseinate is not completely converted into calcium lactate until the fermentation has been going on about 24 hours. Broadly speaking, the calcium in $CaHPO_4$ is acted upon about twice as rapidly as that in calcium caseinate during the first 12 hours under the conditions of our experiments.

It may be added here that determinations of insoluble magnesium were also made. The results show that the insoluble magnesium goes into solution completely in $11\frac{1}{2}$ hours, which is somewhat sooner than the insoluble calcium in $CaHPO_4$.

Effect of the Souring of Milk upon Milk-Albumin.

In Table II the per cent of nitrogen in the milk used in our experiments is given as 0.0762 gm. per 100 cc. of milk; the per cent of nitrogen in forms other than casein and albumin is 0.0498 gm., and the per cent of nitrogen in serum is 0.770 gm. per 100 cc. of milk. The amount of nitrogen in the serum includes that in forms other than casein and albumin, and the amount of this nitrogen subtracted from the nitrogen found as albumin in milk gives the amount of albumin in the serum, which is 0.0272 gm. per 100 cc. of milk. The amounts of total nitrogen and of albumin nitrogen in serum have been determined at intervals during the souring of milk and the results are given in Table VI.

The significant point that attracts attention in connection with the data in Table VI is the increase of nitrogen in the serum. This increase is due to albumin since there was no appreciable solution of casein under the conditions of our work. The formation of lactic acid is apparently responsible for this change. The change may be due to decrease of adsorption of albumin by

casein consequent upon the change produced in casein from caseinate to free casein, or it may be due to a combination of base with albumin in milk and a gradual separation of base and protein in much the same manner as happens with casein.

TABLE VI.

Nitrogen in Serum and Its Relation to Albumin.

Age of milk when sampled.	Total nitrogen in serum from 100 cc. of milk.	Percentage of increase of nitrogen in serum.	Nitrogen as albumin in serum from 100 cc. of milk.	Percentage of albumin of milk in serum.
hrs.	*gm.*		*gm.*	
Fresh.	0.0770	--	0.0272	35.7
10	0.0950	23.4	0.0452	60.0
11	0.0970	26.0	0.0472	62.0
12	0.1120	45.5	0.0622	81.6
13	0.1180	53.2	0.0682	90.0
14	0.1270	65.0	0.0772	100.0

SUMMARY.

1. Fresh milk was analyzed for its soluble and insoluble constituents, a porous porcelain filter being used to make separation. Another portion of the same milk was inoculated with a culture containing *Bacterium lactis acidi* and *Bacterium lactis aerogenes*. At the end of 60 hours determinations were made of the soluble and insoluble portions. (*a*) About 22 per cent of the milk-sugar is changed by the lactic acid bacteria, 88.5 per cent of the amount changed being converted into lactic acid. (*b*) Citric acid completely disappears. (*c*) The insoluble inorganic constituents of the fresh milk are made soluble by the lactic acid. (*d*) Albumin of sour milk passes through the porcelain filter completely. (*e*) Calcium caseinate is changed into free protein and precipitated, the calcium forming lactate.

2. To study the rate and extent of chemical change under given conditions, fresh, pasteurized, separated skim-milk was inoculated with a pure culture of *Bacterium lactis acidi* and kept at 32.2°C. Samples were taken for analysis at intervals during 96 hours. (*a*) Most of the change in milk-sugar occurs between the 10th and 24th hours. When the milk contains 0.7 per cent of lactic acid, the bacterial activity is much reduced. (*b*) The acidity

increases most rapidly during the first 24 hours, the rate of increase diminishing after that. The increased acidity of the serum is due to increase of lactic acid. In the insoluble portion of the milk the free casein is the acid constituent. (c) Calcium combined as $CaHPO_4$ goes into solution completely in 13½ hours. Calcium combined as caseinate is acted upon more slowly, complete solution requiring about 24 hours. (d) The amount of albumin nitrogen in serum increases with increase of acidity; all the albumin of the milk appears in the serum in 14 hours.

THE RATE OF UREA EXCRETION.

A CRITICISM OF AMBARD AND WEILL'S LAWS OF UREA EXCRETION.

By THOMAS ADDIS and C. K. WATANABE.

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Ambard and Weill[1] have reached the conclusion that the rate of excretion of urea by the normal kidney is dependent on only two factors, the concentration of urea in the blood and in the urine. Their method was to collect urine over as short a period of time as was consonant with accuracy in collection, and to compare the rate of urea excretion with the concentration of urea present in the urine and in a specimen of blood removed at about the middle of the period of urine collection. When the concentration of urea in the urine in one individual happened to be the same as that found in another, they noted that the rate of urea excretion varied proportionally to the square of the concentration of urea in the blood. Further, when the blood urea concentration was the same in different individuals, the rate of urea excretion varied inversely as the square root of the urine concentration. These two observations were expressed in mathematical form and combined in a single formula, a correction for body weight being introduced. They showed that the resultant of this formula in all normal people under the most widely different conditions of urea and water intake was a constant. A departure from the constant was taken as indicating an abnormality in kidney function. The formula has been widely used by clinicians—mainly in France—as a means whereby minor deviations from the normal may be detected.

McLean and Selling,[2] and McLean[3] have recently published a large number of observations on the concentration of urea in the blood and urine of normal individuals. The resultant of Ambard's formula in these cases was, they considered, an approximate constant, and they recommend the method for clinical use.

[1] Ambard, L., and Weill, A., *J. physiol. et path. gén.*, 1912, xiv, 753.
[2] McLean, F. C., and Selling, L., *J. Biol. Chem.*, 1914, xix, 31.
[3] McLean, F. C., *J. Exp. Med.*, 1915, xxii, 212.

The two separate laws on which Ambard's formula is based have not yet been confirmed. McLean and Selling, and McLean deal only with the combined formula, and it is noteworthy that the observations on which Ambard and Weill established these empirical laws are very few in number. The main proof that they are correct is allowed to rest on the approximate constancy of the combined formula. The reason for this lies in the fact that in any series of individuals it is only a chance when either the urine concentration or the blood concentration happen to be the same. There is no way to make them the same. Therefore even in a considerable number of observations, there may be relatively few in which this constancy is attained.

It so happened that in the course of an investigation into the factors regulating the rate of urea excretion, which has been carried on for over a year, we had accumulated figures which bear directly on the question of the validity of Ambard ۔ Weill's laws. After the appearance of McLean and Selli۱ ۱ parent confirmation of Ambard and Weill's theories, we th۰ ۱۰°it well to review these data. We found that we had a large number of instances in which either the urine or the blood urea concentration of different individuals was the same or within the limits of error of the methods we employed. These cases have been grouped together in the tables. According to the laws of urea excretion there should be a different constant for each group, to which each instance within the group conforms, when the formulas given by Ambard and Weill are applied. We found that there was no approach to anything that could be even roughly described as a constant. Variations of over 100 per cent were frequent. It is evident from the tables that other factors than the concentration of urea in the blood and urine must commonly intervene in the process of urea excretion.[4]

We believe it is important to demonstrate that the factors governing the excretion of urea by the kidneys are not fully known, for the impression that they are not only known but are measurable with mathematical accuracy is likely to retard further investigation. The approximate constancy of the combined formula, which after all is only roughly approximate, is due in part to the

[4] These tables may seem unnecessarily long, but we expect to refer to them in subsequent communications.

tendency for increased urea concentration in the blood to be accompanied by an increased rate of urea excretion. But in large part it is to be ascribed to its mathematical construction. The more variable factors—the concentration in the urine, the volume of urine, and the amount of urea in the urine—occur as the square or fourth roots of their values. Their disturbing effect on the constancy of the resultant of the formula is thus greatly reduced, while the only factor used without such modification—the concentration in the blood—is itself the most constant quantity used.

Methods.

The urease method of urea estimation was used. With the blood a method almost identical with that of Van Slyke and Cullen was employed, except that during the last few months we have mixed the blood with finely ground soy bean meal, instead of using a purified extract. We never aerated '·   '· than 40 minutes. Most of the estimations recorded here were do.r   ·iplicate. In the urine Marshall's original method was used with twι   ,ιcations. The urine was collected in vessels containing 5 cc. of N ι.ιυυι for every hour's collection, and before adding the urease the contents of the flask were made just alkaline to rosolic acid. The reasons for these changes will be detailed in a paper on urea excretion, which will be published soon. All specimens were diluted to the same volume. The activity of the soy bean extract was determined every day by estimations of known urea solutions. Double determinations were carried out for every specimen, one without and one with the addition of a known amount of urea. The subjects were instructors and students between the ages of 20 and 35, all free from any evidence of disease of the kidneys. The majority underwent an independent investigation of their power to excrete large amounts of urea in which a marked uniformity of functional power was established.

The Relationship between the Concentration of Urea in the Blood and the Rate of Urea Excretion, when the Concentration of Urea in the Urine Is the Same.

The error in the estimation of the concentration of urea in the urine depends on the combined errors in the measurement of the volume of urine and of the amount of urea.

In measuring the volume fractions of 1 cc. were not recorded, so that a volume recorded as 50 cc. might actually be an amount from 49.5 to 50.5 cc. An error of ± 0.5 cc. has therefore been assumed. In regard to the urea estimation we have found, as

Marshall pointed out, that the urease ferment falls a little short of being absolutely quantitative in its action. This, however, is an error in one direction and is relatively constant. The greatest source of error arises from the not very sharp end-points in the titrations. It would seem that an assumed error of ± 1 per cent would certainly be well within the actual limits of error. These combined errors have been adopted as a means of classifying our material. All cases in which the concentration of urine falls within the sum of these limits of error are grouped as having, as far as can be ascertained, the same concentration of urea in the urine. The actually recorded concentration is given for each case. These groups of cases in which the urine concentration is constant have been arranged in order of ascending magnitude of urine concentration, and the cases within the groups in order of descending rates of urea excretion. The rate of urea excretion is expressed as the quantity of urea eliminated in 1 hour, since that was the interval of time during which the collection of urine was made in most instances. When the conditions were such that the volume of urine was large, the time of collection was $\frac{1}{4}$ of an hour, and in some cases when the volume was small, the period of collection was 2 hours. For convenience of reference these cases have been expressed in terms of 1 hour's excretion. In all cases blood was removed from an arm vein at or near the middle of the time of urine collection.

A study of the data thus.given in Table I shows that there is no constant relationship between blood urea concentration and the rate of excretion in cases in which the urine concentration is constant.[5]

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[5] Ambard and Weill's first law states that when the concentration of urea in the urine is constant, the rate of urea excretion varies proportionally to the square of the concentration of urea in the blood, or

$$\frac{(\text{Blood concentration})^2}{\text{Rate of excretion}} = \text{Constant.}$$

The expression of this law which is employed by them is

$$\frac{\text{Blood concentration}}{\sqrt{\text{Rate of excretion}}} = \text{Constant.}$$

This formula has been used by us. It is perhaps worthy of note that it is **to**

There are 135 instances in which a comparison of the blood concentration and the rate of excretion may be made between pairs of cases with the same urine concentration; in 92 (68.1 per cent) the rate of excretion was greater with higher blood concentration, and in 43 (31.8 per cent) the rate of excretion was less. It may be said, then, that in cases where the concentration in the urine is the same, there is a tendency for the rate of excretion to be more rapid when the blood concentration is higher, but at most it is one which is frequently obscured. There are many examples where the opposite seems to hold, as for instance the two cases where one with a blood concentration of 0.0232 per cent excretes 0.98 gm. of urea, while the other with a blood concentration of 0.0450 per cent eliminates only 0.39 gm.

The groups in Table I comprise examples of wide variation in rate of urea excretion corresponding to differences in intake of urea or of urea-forming food. It was conceivable that under uniform conditions it might be possible to establish a more evident connection between blood concentration and rate of urea excretion. In Table II only those subjects are included who were under the same conditions as regards nitrogen and water consumption. Here also, however, it will be noted that there are cases where a greater rate of urea excretion is associated with a lower percentage of blood urea.

some extent this particular mathematical form which is responsible for the relatively smaller percentage variation in the constants for the cases within the various groups seen in Table I as compared with those in Table III. The rate of excretion is the most variable of all the factors, and when it is reduced to its square root an appearance of greater uniformity is obtained. In the combined formula a correction for body weight is introduced, on the assumption that a heavy man will have a higher rate of excretion than a light one, other things being equal. We have introduced this factor in the same manner as used by Ambard and Weill, expressing the body weight in relation to the standard weight of 70 kg. Further, in order to make our formula identical with theirs, we have converted our hourly rates of excretion into 24 hour quantities, and our blood concentrations into gm. per liter. The formula then reads

$$\frac{\text{Gm. of urea per liter of blood}}{\sqrt{\text{Hourly rate of excretion} \times 24 \times \dfrac{70}{\text{Kg. of body weight}}}} = \text{Constant}.$$

*The Relationship between the Concentration of Urea in the Urine
and the Rate of Urea Excretion when the Concentration
of Urea in the Blood Is the Same.*

In 90 duplicate estimations of blood·urea there was an average
variation of 1.5 mg.[6] The greatest variation was 6.6 mg. The
results recorded are not, however, the average of two determi-
nations, but the higher of two. The reason for this is that, apart
from overtitration, positive errors are unlikely. If any alkali were
blown over there would be a large positive error, but we have
never seen this occur. It appears to us that the cause of variation
in duplicate estimations, apart from the titration error, is due
in practically all cases to incomplete decomposition of urea or to
incomplete removal of ammonia. Perhaps the physical conditions
in the mixture of blood, alkali, and soy bean powder or extract
may sometimes be such as either to protect a trace of urea from the
action of the ferment, or to hinder a little ammonia from being
carried over in the air current, since a variable degree of clumping
and agglutination of the material is often observed at the com-
mencement of the aeration and towards its close.

We cannot say what the actual error is, but this is immaterial.
since we require to fix a degree of variation which is certainly
well within the actual limits of error. We have arbitrarily chosen
0.6 mg. as corresponding to this requirement. All cases in which
the difference in blood concentration was not greater than 0.6 mg.
have therefore been grouped together. The same arrangement of
the groups and of the cases within the groups has been adopted as
in Table I.

The figures in Table III show that there is no constant relation-
ship between the concentration of urea in the urine and the rate
of urea excretion in cases in which the blood concentration is the
same.[7]

[6] In a large series of duplicate estimations carried out since these were
completed, the average error has been only 0.5 mg.

[7] Ambard's second law of urea excretion states that when the concentra-
tion of urea in the blood is the same, the rate of urea excretion varies in-
versely as the square root of the concentration of urea in the urine, or,

$$\text{Rate of excretion} \times \sqrt{\text{Urine concentration}} = \text{Constant.}$$

The group constants have been worked out in the same way as for Table
I, introducing the correction for body weight and converting the con-

In 136 comparisons between pairs of cases with the same blood concentration there were 91 instances (65.9 per cent) in which the rate of excretion was less with higher urine concentration, and 45 instances (34.0 per cent) in which it was greater with higher urine concentration. Here again all that can be said is that there appears to be a tendency for the rate of urea excretion to be slowed with increased urine concentration in cases in which the blood concentration is the same.

In Table IV subjects with the same blood concentration who were on a diet with the same nitrogen and water content have been grouped. No marked difference from Table III is apparent.

There are a number of instances where both the urine and blood urea concentrations are the same in different individuals or in the same individual at different times. If Ambard and Weill's theories are correct, these cases should show the same rate of urea excretion. They have been grouped in Table V. Wide variations in the rate of urea excretion are seen in these cases. There could not be any clearer indication than this that other factors besides urea concentration are responsible for the rate of urea excretion.

CONCLUSION.

The rate of urea excretion in man varies under physiological conditions in a manner which cannot be explained by the concentrations of urea in the blood and urine.

There is a tendency for an increased rate of urea excretion to exist with higher blood urea concentration in cases in which the urine concentration is the same, and for an increased rate of urea excretion to be accompanied by a lowering of the urea concentration in the urine in cases in which the blood concentration is the same.

This relationship, however, is one which is frequently obscured, even in individuals under the same conditions as regards nitrogen and water intake.

Other factors than urea concentration are important in determining the rate of urea excretion by the normal kidney.

centration of urea in the urine into gm. per liter. The formula therefore reads

$$\text{Hourly rate of excretion} \times 24 \times \dfrac{70}{\text{Kg. of body weight}} \times$$

$$\dfrac{}{\sqrt{\text{Gm. of urea per liter of urine}}} = \text{Constant.}$$

TABLE I.

Groups of Cases in Which the Concentration of Urea in the Urine Was the Same.

Subject.	Weight factor, 70 kg. Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
Boy............	0.91	0.155	0.0324	760	1.18	0.0639
Add...........	1.05	0.157	0.0402	600	0.94	0.0826
Add...........	1.05	0.171	0.0414	640	1.096	0.0787
Add...........	1.05	0.172	0.0354	634	1.094	0.0675
Add...........	1.05	0.180	0.0300	632	1.14	0.0560
Add...........	1.05	0.181	0.0312	596	1.08	0.0598
Boy............	0.91	0.178	0.0336	584	1.04	0.0706
Ca.............	0.84	0.197	0.0450	922	1.81	0.0746
Add...........	1.05	0.196	0.0408	688	1.35	0.0700
J..............	1.14	0.198	0.0399	590	1.17	0.0701
Mo............	0.84	0.198	0.0384	508	1.00	0.0855
Ca.............	0.84	0.205	0.0384	822	1.81	0.0635
Add...........	1.05	0.202	0.0489	600	1.21	0.0884
Mo............	0.84	0.215	0.0402	508	1.095	0.0855
Mo............	0.84	0.219	0.0402	496	1.088	0.0855
V..............	0.95	0.63	0.0225	151	0.96	0.0482
Add...........	1.05	0.64	0.0300	143	0.91	0.0636
K..............	1.03	1.00	0.0216	93	0.93	0.0450
Cr.............	1.06	1.00	0.0210	75	0.75	0.0482
B..............	1.11	1.06	0.0240	87	0.93	0.0482
K..............	1.03	L.04	0.0337	84	0.87	0.0728
V..............	0.95	1.16	0.0225	64	0.74	0.0550
O..............	1.09	1.16	0.0204	62	0.72	0.0470
V..............	0.95	1.17	0.0270	51	0.60	0.0730
G..............	0.94	1.14	0.0288	34	0.39	0.0970
Ca.............	0.84	1.25	0.0390	83	1.03	0.0857
Bo.............	0.81	1.27	0.0261	73	0.93	0.0614
K..............	1.03	1.28	0.0246	56	0.72	0.0581
We............	1.13	1.25	0.0180	42	0.52	0.0482

TABLE I—*Continued*.

Subject.	Weight factor, 70 kg. Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
Ca............	0.84	1.30	0.0367	81	1.05	0.0718
Add...........	1.05	1.29	0.0322	80	1.03	0.0631
K.............	1.03	1.31	0.0216	43	0.57	0.0578
Ch............	1.09	1.35	0.0379	121	1.35	0.0638
M.............	1.08	1.34	0.0156	29	0.39	0.0490
M.............	1.08	1.43	0.0558	216	3.09	0.0624
Ca............	0.84	1.45	0.0292	90	1.30	0.0571
Add..........	1.05	1.44	0.0322	44	0.63	0.0807
Add..........	1.05	1.59	0.0585	107	1.71	0.0892
Add..........	1.05	1.58	0.0472	53	0.84	0.1025
Add..........	1.05	1.63	0.0540	79	1.29	0.0945
J.............	1.14	1.62	0.0252	52	0.84	0.0526
Sh............	1.00	1.65	0.0330	37	0.61	0.0863
C.............	0.93	1.71	0.0246	56	0.96	0.0533
K.............	1.03	1.71	0.0360	47	0.80	0.0812
F.............	0.90	1.74	0.0240	42	0.73	0.0605
Wi............	1.08	1.72	0.0246	34	0.58	0.0634
Add..........	1.05	1.71	0.0276	28	0.48	0.0795
Sh............	1.00	1.73	0.0292	27	0.47	0.0870
Ch............	1.09	1.73	0.0252	26	0.45	0.0737
K.............	1.03	1.78	0.0307	39	0.69	0.0745
Sh............	1.00	1.77	0.0337	23	0.41	0.1072
Bb............	0.81	1.87	0.0300	47	0.88	0.0724
Add..........	1.05	1.91	0.0322	45	0.86	0.0692
Wa............	1.58	1.90	0.0250	32	0.61	0.0520
Add..........	1.05	1.97	0.0577	109	2.14	0.0789
Add..........	1.05	1.99	0.0495	104	2.06	0.0695
Mc............	1.19	1.95	0.0246	50	0.97	0.0468
Sh....	1.00	1.94	0.0330	24	0.47	0.0984
Add..........	1.05	1.95	0.0315	20	0.39	0.1002
Add..........	1.05	2.01	0.0465	60	1.21	0.0841
S.............	1.08	2.00	0.0240	30	0.60	0.0610

TABLE I—*Continued.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
Add........	1.05	2.06	0.0765	121	2.49	0.0966
W........	1.11	2.08	0.0492	118	2.46	0.0607
Add........	1.05	2.05	0.0367	51	1.04	0.0717
Ai........	1.19	2.07	0.0282	39	0.81	0.0585
Add........	1.05	2.06	0.0300	18	0.37	0.0980
B........	1.11	2.23	0.0516	84	1.87	0.0733
Mo........	0.82	2.22	0.0485	48	1.07	0.1053
Ad........	0.99	2.21	0.0438	43	0.95	0.0923
Add........	1.05	2.23	0.0427	41	0.91	0.0892
P........		2.21	0.0282	30	0.67	
O........	1.09	2.34	0.0546	110	2.58	0.0665
Add........	1.05	2.35	0.0346	51	1.20	0.0630
Mo........	0.82	2.34	0.0232	42	0.98	0.0529
Add........	1.05	2.33	0.0450	17	0.39	0.1430
Add........	1.05	2.38	0.0495	71	1.70	0.0755
Mo........	0.82	2.42	0.0271	44	1.07	0.0590
Add........	1.05	2.46	0.0442	39	0.96	0.0899
Br........	1.01	2.40	0.0252	20	0.48	0.0740
Ch........		2.61	0.0435	68	1.77	
So........	0.98	2.64	0.0427	40	1.05	0.0860
Mo........	0.82	2.68	0.0435	38	1.02	0.0970
Jo........	1.14	2.63	0.0375	37	0.97	0.0726
Add........	1.05	2.78	0.0682	78	2.17	0.0925
Add........	1.05	2.75	0.0607	69	1.89	0.0881
C........	0.93	3.15	0.0624	87	2.74	0.0805
Do........	1.00	3.23	0.0651	80	2.59	0.0828
Boy........	0.91	3.22	0.0590	72	2.32	0.0830
Cr........	1.06	3.21	0.0558	70	2.25	0.0737
K........	1.03	3.15	0.0672	68	2.14	0.0925
T........	0.86	3.15	0.0252	48	1.51	0.0451
Jo........	1.14	3.31	0.0397	25	0.828	0.0835
Jo........	1.14	3.29	0.0397	23	0.756	0.0874

TABLE I—*Concluded.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urine excreted in 1 hr.	Constants by Ambard's first law.
		gm.	*gm.*	*cc.*	*gm.*	
E..............	1.08	3.55	0.1230	101	3.60	0.1270
Wa............	1.58	3.56	0.0678	69	2.46	0.0704
Ad............	0.90	3.54	0.0750	68	2.41	0.0992
F..............	0.90	3.65	0.0558	66	2.41	0.0774
G..............	0.94	3.63	0.0612	52	1.89	0.0935
Bo............	1.03	3.72	0.0672	58	2.16	0.0920
Ai.............	1.19	3.69	0.0690	58	2.14	0.0885
So............	0.98	3.71	0.0382	20	0.74	0.0917
Jo............	1.14	3.72	0.0405	19	0.71	0.0920
J..............	1.10	4.00	0.0630	62	2.47	0.0816
Wa............	1.58	4.08	0.0630	58	2.37	0.0664
T..............	0.86	4.22	0.0900	104	4.41	0.0945
K..............	1.03	4.33	0.1050	91	3.96	0.1065
Bo............		4.85	0.0980	89	4.32	
P..............		4.89	0.0732	51	2.52	

TABLE II.

The 3rd Day of a Diet Constant in Nitrogen and Water.   Groups of Ca‹
Which the Concentration of Urea in the Urine Was the Same.

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Cons b Aml first
		gm.	gm.	cc.	gm.	
K.............	1.03	1.00	0.0216	93	0.93	0.
C.............	1.06	1.00	0.0210	75	0.75	0.
Bo............	0.81	1.27	0.0261	73	0.93	0.
K.............	1.03	1.28	0.0246	56	0.72	0.
We...........	1.13	1.25	0.0180	42	0.52	0.
K.............	1.03	1.31	0.0216	43	0.57	0.
M............	1.09	1.34	0.0156	29	0.39	0.
C.............	0.93	1.71	0.0246	56	0.96	0.
F.............	0.90	1.74	0.0240	42	0.73	0.
Wi............	1.08	1.72	0.0246	34	0.58	0.
Add..........	1.05	1.71	0.0276	28	0.48	0.
Ch............	1.09	1.73	0.0252	26	0.45	0.
Bo............	0.81	1.87	0.0300	47	0.88	0.
Wa...........	1.58	1.90	0.0250	32	0.61	0.
McC..........	1.19	1.95	0.0246	50	0.97	0.
S.............	1.08	2.00	0.0240	30	0.60	0.
Ad............		2.21	0.0438	43	0.95	
P.............		2.21	0.0282	30	0.67	
D.............	1.00	2.29	0.0312	38	0.87	0.
Ma...........		2.31	0.0210	22	0.51	

The 6th Day of the Same Diet with 20 Gm. of Urea n 360 Cc. of Water T
on the 4th, 5th, and 6th Days

C.............	0.93	3.15	0.0624	87	2.74	0.
D.............	1.00	3.23	0.0651	80	2.59	0.
Bo............	0.91	3.22	0.0590	72	2.32	0.
Cr............	1.06	3.21	0.0558	70	2.25	0.
K.............	1.03	3.15	0.0672	68	2.14	0.
Add..........	1.05	3.56	0.0678	69	2.46	0.
Ad............	0.99	3.54	0.0750	68	2.41	0.
F.............	0.90	3.65	0.0558	66	2.41	0.
G.............	0.95	3.63	0.0612	52	1.89	0.
Bo............	0.81	3.72	0.0672	58	2.16	0.
Ai............	1.19	3.69	0.0690	58	2.14	0.
J.............	1.01	4.00	0.0630	62	2.47	0.
Wa...........	1.58	4.08	0.0630	58	2.37	0.

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TABLE III.

Groups of Cases in Which the Concentration of Urea in the Blood Was the Same.

Subject.	Weight factor, 70 kg. Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
K _ _ _ _	1.03	0.0216	1.00	92	0.93	72.5
W _ _ _	1.13	0.0210	0.94	82	0.77	64.1
A_ _ _ _	1.06	0.0210	1.00	75	0.75	60.4
K _ _ _ _	1.03	0.0216	1.31	43	0.57	51.0
M _ _ _	1.09	0.0210	2.31	22	0.51	68.2
V_ _ _ _	0.95	0.0225	0.63	151	0.96	54.8
V_ _ _ _	0.95	0.0225	1.16	64	0.74	57.6
McC	1.19	0.0246	1.95	50	0.97	122.2
C_ _ _ _	0.93	0.0246	1.71	56	0.96	88.9
B_ _ _ _	1.11	0.0240	1.06	87	0.93	80.6
F_ _ _ _	0.90	0.0240	1.74	42	0.73	65.7
K _ _ _ _	1.03	0.0246	1.28	56	0.72	63.6
S_ _ _ _	1.08	0.0240	2.00	30	0.60	69.6
Wi_ _ _	1.08	0.0246	1.73	34	0.58	62.5
T...........	0.86	0.0252	3.15	48	1.51	175.0
J...........	1.01	0.0252	1.62	52	0.84	82.0
Wa_ _	1.58	0.0250	1.90	32	0.61	101.0
Br_	1.01	0.0252	2.40	20	0.48	57.1
Ch	1.09	0.0252	1.73	26	0.45	48.9
Mo_ _	0.82	0.0271	2.42	44	1.07	103.5
V_ _ _	0.95	0.0270	1.17	51	0.60	46.9
Add _	1.05	0.0276	1.71	28	0.48	50.0
Li_ _ _	1.19	0.0282	2.07	39	0.81	105.0
a_ _		0.0282	2.21	30	0.67 ·	
_ _ _ _	0.94	0.0288	1.14	34	0.39	29.9
L_ _ _	0.84	0.0292	1.45	90	1.31	100.5
_ _ _	1.00	0.0292	1.73	27	0.47	46.9
d	1.05	0.0300	0.18	732	1.14	38.6
d	1.05	0.0300	0.64	143	0.91	58.0
_	0.81	0.0300	1.87	47	0.88	74.0
d	1.05	0.0300	2.06	18 ·	0.37	42.4

TABLE III—*Continued*.

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constant by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
Add...........	1.05	0.0312	0.18	596	1.08	36.6
D.............	1.00	0.0312	2.29	38	0.87	100.0
Add...........	1.05	0.0315	1.95	20	0.39	43.1
Boy...........	0.91	0.0324	0.15	760	1.18	31.5
Add...........	1.05	0.0322	1.29	80	1.03	93.2
Add...........	1.05	0.0322	1.91	45	0.86	94.5
Add...........	1.05	0.0322	0.77	112	0.85	59.5
Add...........	1.05	0.0322	1.47	44	0.64	61.8
Add...........	1.05	0.0322	0.97	54	0.52	40.7
Sh............	1.00	0.0330	1.65	37	0.61	59.5
Sh............	1.00	0.0330	1.94	24	0.47	52.2
Add...........	1.05	0.0336	0.19	572	1.10	38.3
Boy...........	0.91	0.0336	0.18	584	1.04	30.5
K.............	1.03	0.0337	1.04	84	0.88	70.1
Add...........	1.05	0.0337	0.44·	181	0.81	42.0
Sh............	1.00	0.0337	1.77	23	0.41	42.0
Ca............	0.84	0.0360	0.91	127	1.15	70.0
K.............	1.03	0.0360	1.71	47	0.80	81.9
Ca............	0.84	0.0367	1.30	81	1.05	76.5
Add...........	1.05	0.0367	2.05	51	1.04	· 118.4
Ch.	1.09	0.0379	1.35	121	1.36	130.8
Jo............	1.14	0.0375	2.63	37	0.97	135.8
Ca............	0.84	0.0384	0.20	882	1.81	51.5
T.............	1.01	0.0384	0.15	718	1.05	31.2
Mo............	0.82	0.0384	0.20	508	1.00	27.9
So............	0.98	0.0382	3.71	20	0.74	106.0
T.............	1.01	0.0390	0.14	750	1.09	31.2
Ca............	0.84	0.0390	1.30	81	1.06	77.0
J.............	1.01	0.0398	0.20	590	1.17	40.2
Jo............	1.14	0 0397	3.31	25	0.83	131.5
Jo............	1.14	0.0397	3.29	23	0.76	119.0

TABLE III—*Continued.*

Subject.	Weight factor, 70 kg. Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
Add..........	1.05	0.0408	0.20	688	1.35	48.1
Mo..........	0.82	0.0402	0.21	508	1.09	31.0
Mo..........	0.82	0.0402	0.22	496	1.08	31.5
Add..........	1.05	0.0402	0.16	600	1.04	33.2
Jo..........	1.14	0.0405	3.72	19	0.71	118.8
Add..........	1.05	0.0414	0.72	196	1.30	87.9
Add..........	1.05	0.0414	0.17	640	1.09	35.9
Add..........	1.05	0.0427	0.92	193	1.78	136.4
Add..........	1.05	0.0426	0.23	704	1.60	61.2
So..........	0.98	0.0427	2.64	40	1.06	128.0
So..........	0.98	0.0427	3.51	27	0.94	131.0
Add..........	1.05	0.0427	2.23	41	0.91	108.1
So..........	0.98	0.0427	3.82	21	0.80	116.2
Ch..........	1.09	0.0435	2.61	68	1.77	236.5
Mo..........	0.82	0.0438	0.52	292	1.52	68.0
Mo..........	0.82	0.0435	2.68	38	1.02	104.0
Ad..........	0.99	0.0438	2.21	43	0.95	106.0
Ca..........	0.84	0.0450	0.20	922	1.81	67.0
Add..........	1.05	0.0450	2.33	17	0.40	48.7
Mo..........	0.82	0.0462	2.04	78	1.60	142.2
Add..........	1.05	0.0465	2.01	60	1.21	137.0
Add..........	1.05	0.0473	0.92	193	1.78	136.3
Add..........	1.05	0.0472	1.58	53	0.84	84.0
Add..........	1.05	0.0489	0.20	600	1.21	43.1
Mo..........	0.82	0.0485	2.22	48	1.07	99.4
Add..........	1.05	0.0495	1.99	104	2.06	231.8
Add..........	1.05	0.0495	2.38	71	1.70	209.5
Add..........	1.05	0.0514	2.53	79	2.00	253.8
B..........	1.11	0.0516	2.23	84	1.87	235.9
Add..........	1.05	0.0517	0.92	193	1.78	136.1
O..........	1.09	0.0546	2.34	110	2.58	329.8
Add..........	1.05	0.0540	1.95	51	1.00	111.3

TABLE III—*Concluded.*

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
M..............	1.08	0.0558	1.43	216	3.09	304.1
F..............	0.90	0.0558	3.65	66	2.41	316.0
Cr.............	1.06	0.0558	3.21	70	2.25	324.0
S..............	1.08	0.0606	2.87	72	2.07	287.8
Add...........	1.05	0.0607	2.75	69	1.90	251.6
G..............	0.94	0.0612	3.63	52	1.89	258.0
C..............	0.93	0.0624	3.15	87	2.74	344.0
J..............	1.01	0.0630	4.00	62	2.47	379.5
Wa............	1.58	0.0630	4.08	58	2.37	570.0
Bo.............	1.03	0.0672	3.72	58	2.16	326.0
K..............	1.03	0.0672	3.15	68	2.14	296.8
Add...........	1.05	0.0678	3.56	69	2.46	370.2
Add...........	1.05	0.0682	2.78	78	2.17	289.0

TABLE IV.

Groups of Cases in Which the Concentration of Urea in the Blood Was the Same.

Subject.	Weight factor, 70 kg. / Kg. of body weight.	Blood concentration per 100 cc.	Urine concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.	Constants by Ambard's second law.
		gm.	*gm.*	*cc.*	*gm.*	
K	1.03	0.0216	1.00	92	0.93	72.5
Cr	1.06	0.0210	1.00	75	0.75	60.4
K	1.03	0.0216	1.31	43	0.57	51.0
Ma		0.0210	2.31	22	0.51	
McC	1.19	0.0246	1.95	50	0.97	122.2
C	0.93	0.0246	1.71	56	0.96	88.9
B	1.11	0.0240	1.06	87	0.93	80.6
F	0.90	0.0240	1.74	42	0.73	65.7
K	1.03	0.0246	1.28	56	0.72	63.6
S	1.08	0.0240	2.00	30	0.60	69.6
Wi	1.08	0.0246	1.73	34	0.58	62.5
T	0.86	0.0252	3.15	48	1.51	175.0
J	1.01	0.0252	1.62	52	0.84	82.0
Wa	1.58	0.0250	1.90	32	0.61	101.0
Br	1.01	0.0252	2.40	20	0.48	57.1
Ch	1.09	0.0252	1.73	26	0.45	48.9
Ai	1.19	0.0282	2.07	39	0.81	105.0
P		0.0282	2.21	30	0.67	
Add	1.05	0.0276	1.71	28	0.48	50.0

The 6th Day of the Same Diet with 20 Gm. of Urea in 360 Cc. of Water Taken on the 4th, 5th, and 6th Days.

Subject						
We	1.13	0.0510	2.57	84	2.16	297.0
B	1.11	0.0516	2.23	84	1.87	235.9
Me	1.09	0.0558	1.43	216	3.09	304.1
F	0.90	0.0558	3.65	66	2.41	316.0
Cr	1.06	0.0558	3.21	70	2.25	324.0
S	1.08	0.0606	2.87	72	2.07	287.8
Br	1.01	0.0600	5.14	35	1.80	313.0
C	0.93	0.0624	3.15	87	2.74	344.0
T	1.01	0.0630	4.00	62	2.47	379.0
Wa	1.58	0.0630	4.08	58	2.37	570.0
Add	1.05	0.0678	3.56	69	2.46	370.2
Bo	1.03	0.0672	2.16	58	2.16	326.0
K	1.03	0.0672	3.15	68	2.14	296.8

TABLE V.

Groups of Cases in Which the Concentration of Urea in the Urine and in the Blood Was the Same.

Subject.	Weight factor, $\frac{70 \text{ kg.}}{\text{Kg. of body weight.}}$	Urine concentration per 100 cc.	Blood concentration per 100 cc.	Volume of urine in 1 hr.	Urea excreted in 1 hr.
		gm.	*gm.*	*cc.*	*gm.*
Ca.......	0.84	0.20	0.0384	882	1.81
Mo.......	0.82	0.20	0.0384	508	1.00
Mo.......	0.84	0.21	0.0402	508	1.09
Mo.......	0.84	0.21	0.0402	496	1.08
K........	1.03	1.00	0.0216	93	0.93
Cr.......	1.06	1.00	0.0210	75	0.75
C........	0.93	1.71	0.0246	56	0.96
Ch.......	1.09	1.73	0.0252	26	0.45
F........	0.90	1.74	0.0240	42	0.73
Wi.......	1.08	1.72	0.0246	34	0.58
Mc.......	1.19	1.95	0.0246	50	0.97
S.........	1.08	2.00	0.0240	30	0.60
Mo.......	0.82	2.04	0.0462	78	1.60
Add......	1.05	2.01	0.0465	60	1.21
Ch.......	1.09	2.61	0.0435	68	1.77
Mo.......	0.82	2.68	0.0435	38	1.02
Jo........	1.14	3.31	0.0397	25	0.83
Jo........	1.14	3.29	0.0397	23	0.75
J........	1.01	4.00	0.0630	62	2.47
Wa.......	1.58	4.08	0.0630	58	2.37

THE INCREASE OF NITROGEN IN FERMENTING MANURES.*

By W. E. TOTTINGHAM.

(From the Wisconsin Agricultural Experiment Station, Madison.)

Various investigations which have been made concerning the changes of composition in fermenting stored farm manure have nearly always shown a greater or less loss of nitrogen. Such investigations usually have covered periods of fermentation ranging from 2 to 3 months to several months in length. As far as the writer is aware, very few investigators[1] have observed a gain of nitrogen.

In the course of an investigation of the changes in a fermenting mixture of cow and horse manure, with and without litter in the form of barley straw, the writer found gains in the absolute amount of nitrogen after 2 weeks and after 4 weeks of fermentation. Later there were decreases of nitrogen of such extent that after 12 weeks of fermentation the manures showed losses in the absolute amount of nitrogen present. The gain of nitrogen in this experiment was much greater in the straw-littered manure than in the control, unlittered manure. This phenomenon has received further attention, with the results reported in this paper. Acknowledgment is made of the necessary bacteriological assistance rendered by Dr. E. B. Fred of this Experiment Station.

The check experiment was conducted in the usual manner, as follows: Fresh cow manure and fresh horse manure, each free from any but fine litter, were mixed in the ratio of 2: 1. Chopped wheat straw was sifted into two portions one of which passed

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[1] Löhnis, F., Handb. landw. Bakteriol., Berlin, 1910, 745. Henry, J. agr. pratique, 1897, lxi, 411 and 485, Abstracts. Hornberger, Centr. Bacteriol., 2te Abt., 1905, xiv, 423; 1907, xix, 340, Abstracts.

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through a sieve of $\frac{5}{16}$ inch mesh, but was retained by a sieve of $\frac{1}{4}$ inch mesh. The other portion contained the straw which passed through the finer of the two sieves mentioned. Three portions of the mixed manure amounting to 6.25 kg. each were weighed. Lot 1 was left untreated as a control, Lot 2 received 0.5 kg. of the coarser straw litter, and Lot 3 received an equal amount of the finer straw. Each lot of manure was next thoroughly mixed and sampled for chemical and bacteriological analysis. It was then weighed in a loosely covered pail of galvanized iron and set aside in a warm room. The weighings were performed on a solution scale sensitive to 1 gm., or to within a possible error of 0.02 per cent for the weight involved. After a period of 4 weeks each lot of manure was weighed again, thoroughly mixed, and sampled as before for analysis. The data of the analyses follow:

TABLE I.

		Fresh weight.	Dry matter.		Loss of dry matter.	Nitrogen in dry matter.		Gain (+) or loss (−) of nitrogen.	Bacteria in millions per gm. of dry matter.
		gm.	per cent	gm.	per cent	per cent	gm.	per cent	
Lot 1. Control, manure alone...	At beginning......	5,489	20.3	1,115	—	1.67	18.60	—	5 -
	After 4 wks.	5,281	17.1	904	18.92	1.95	17.63	−5.22	110 -
Lot 2. Manure with coarser wheat straw...	At beginning......	6,095	25.0	1,524	—	1.38	21.03	—	53 -
	After 4 wks.	5,710	18.2	1,039	31.82	2.12	22.03	+4.76	167 -
Lot 3. Manure with finer wheat straw...	At beginning......	6,118	25.2	1,540	—	1.40	21.56	—	25 -
	After 4 wks.	5,756	18.5	1,064	30.91	2.10	22.36	+3.71	210 -

The data show that there was an appreciable gain of nitrogen in the straw-littered manures. The gain was not so great as that

which had been obtained in the previous experiment in which barley straw had been used. It may be stated that the determinations of dry matter were made on triplicate samples of 100 gm. each, nitrogen was determined in either triplicate or quadruplicate by the Gunning method on 10 gm. samples of the moist manures, and the bacterial counts were made upon agar media.

The different manures were next tested for nitrogen fixation in mannite solution. It was not possible to identify *Azotobacter* forms among the organisms in the manures. Chromogenic forms were predominant among the bacteria which developed on agar plates from the straw-littered manures. The nature of the or-

TABLE II.

	Nitrogen.	Average.	Relative amount of nitrogen with control as 100.
	mg.	*mg.*	
Control,	Flask A, 1.1		
uninoculated.	" B, 1.0	1.05	100
Lot 1. Inoculated	" A, 0.5		
by extract of manure alone.	" B, 0.6	0.55	52
Lot 2. Inoculated	" A, 8.3		
by extract of manure with			
coarser wheat straw.	" B, 12.1	10.20	970
Lot 3. Inoculated	" A, 12.7		
by extract of manure with			
finer wheat straw.	" B, 10.5	11.60	1,100

ganism or organisms responsible for the fixation observed here is receiving attention under the direction of Dr. Fred.

Quantitative measurements of the fixing power of these manures were made by both weak and strong inoculations. The former was conducted as follows: 20 gm. of manure sample were diluted with 400 cc. of sterile water and 10 cc. portions of the dilution were inoculated into two 100 cc. portions of Ashby's solution in large Erlenmeyer flasks. Two similar flasks were treated immediately with 10 cc. of concentrated sulfuric acid, to serve as controls. After 3 weeks' incubation the eight flasks thus derived were analyzed for total nitrogen with the results given in Table II.

The results agree with those of the fermentation, showing presence of nitrogen-fixing forms in the littered manures. ' strong inoculation was conducted as follows: 10 gm. samples fi each lot of manure were added to each of four flasks of Ashl solution. Two flasks of each lot were immediately treated w 10 cc. of concentrated sulfuric acid. The twelve flasks w then incubated for 4 weeks and the contents analyzed for t nitrogen with the results given in Table III.

TABLE III.

		Nitrogen.	Aver-age.	Gain in nitr in unacidi cultures
		mg.	mg.	mg.	pea
Lot 1.	Manure alone.				
	Acidified.	Flask A, 33.7			
		" B, lost.	33.7		
	Unacidified.	" A, 45.5			
		" B, 44.8	45.2	11.5	3
Lot 2.	Manure with coarser wheat straw.				
	Acidified.	" A, 38.2			
		" B, 37.7	38.0		
	Unacidified.	" A, 51.0			
		" B, 48.3	49.7	11.7	3
Lot 3.	Manure with finer wheat straw.				
	Acidified.	" A, 39.0			
		" B, 39.3	39.2		
	Unacidified.	" A, 49.2			
		" B, 47.3	48.3	9.1	2

The results show that the manures contain nitrogen-fix organisms of considerable activity. As their activity was great in the fermentation experiments in those manures which w mixed with straw the latter material appears to be an import source of energy for the organisms.

It is apparent that the gain of nitrogen by manures in the ea stages of fermentation may assume practical importance by production of increased growth in crops unless it is counterl anced by the loss of organic matter which occurs simultaneou Through the courtesy of Professor E. B. Hart of this laboratory writer is privileged to use data from field plots to investigate t

point. Manure is applied to these plots once during a three course *rotation* of corn, barley, and clover, application preceding the corn *crop.* Data covering 6 years show 5.3 per cent greater yield of *corn* grain and 7.4 per cent greater yield of stover from stall *manure* with straw as compared with fresh manure with straw. *This* difference disappears with the barley crop and is reversed *with* the clover crop. The stall manure is taken from the same *supply* as the fresh manure, but is allowed to stand in a covered *box* out of doors during the winter months, so that fermentation is *limited.*

From the results of this limited number of crops it is evident *that* increased value of the manure has resulted from restricted *fermentation,* the nitrogen so fixed becoming available during the *season* of application. Further work is necessary to determine *how* generally applicable the principle may become in the practical *use* of farm manure.

THE DETERMINATION OF CHOLESTEROL IN BLOOD.

BY W. R. BLOOR

(*From* the Laboratories of Biological Chemistry of the Harvard Medical
School, Boston.)

The probable importance of a knowledge of the variations in
the cholesterol content of the blood in pathological conditions has
been recognized for some time; and recently the variations of cho-
lesterol in the normal blood in relation to its function in normal
metabolism have been receiving attention. The earlier methods
put forward for the determination of cholesterol in blood were
gravimetric, requiring relatively large amounts of blood and
considerable expenditure of time, and were therefore unsuited to
the study of changes in the cholesterol content of the blood of the
living organism. The more recent methods[1]—mainly colori-
metric—are better suited to the purpose, since they require only
a few cc. of blood and can be carried out in a short time. Of these
methods the one which appears to have given the most general
satisfaction is that of Autenrieth and Funk,[1] a colorimetric method
depending on the Liebermann-Burchard color reaction. In this
method 2 cc. of blood or plasma are digested for 2 hours on the
water bath with strong alkali, the cholesterol is extracted from the
digestion mixture by repeated shaking with ether or chloroform,
and determined colorimetrically by treatment in chloroform solu-
tion with acetic anhydride and sulfuric acid. Aside from the
long digestion, the inconvenient feature of this method is the
extraction, which requires a total of 25 to 30 minutes of constant
hard shaking together with five or six separations. These extrac-
tions when performed by hand are very tedious. Other objec-
tions to the method as carried out are: (1) the difficulty of ex-

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[1] For a discussion of the methods for cholesterol determination in blood
the reader is referred to Autenrieth, W., and Funk, A., *Münch. Med. Woch.,*
1913, lx, 1243.

tracting cholesterol from strongly alkaline aqueous solutions which was pointed out by Corper;[2] and (2) the fact that the extract frequently contains something which gives a brownish tint to the green color produced in the chloroform and renders the readings difficult. The occurrence of the brownish tint was admitted by Autenrieth and Funk who claimed, however, that i did not interfere with the readings. Klein and Dinkin[3] noted the occurrence of the brown tint and state, on the contrary, that i does interfere with the determination. They ascribe the tint to the action of the strong alkali.

In earlier work on blood lipoids it has been found that the ex traction of the lipoid material from the blood by treatment with alcohol-ether, as previously described,[4] is practically complete and since the time required is short and the treatment relatively gentle it was believed that it offered a good means for the de termination of cholesterol. Attempts were therefore made to apply the Autenrieth-Funk procedure to the determination o cholesterol in this extract. The procedure as finally worked out while yielding higher results than that of Autenrieth and Funk is much more simple and rapid and is therefore believed to be a considerable improvement over the latter.

An attempt was first made to use the untreated alcohol-ether extract of the blood. It was evaporated to dryness, the choles terol taken up with chloroform, and the color produced as usual The chloroform extract was, however, so much tinged with brown that the readings were always doubtful. To obviate the color difficulty the blood extract was next saponified by evap oration to dryness with sodium ethylate and the cholesterol ex tracted from the dry material by boiling out with several portions of chloroform. But although the extracts so obtained were color less, the time required was so great that very little advantage was gained. It was then discovered that if the evaporation of the untreated alcohol-ether extract was carried out carefully so as to avoid heating the residue after it had reached dryness, the chloro form extract was colorless and after treatment gave excellen readings.

[2] Corper, H. J., *J. Biol. Chem.*, 1912, xi, 27.
[3] Klein, W., and Dinkin, L., *Z. physiol. Chem.*, 1914, xcii, 302.
[4] Bloor, W. R., *J. Biol. Chem.*, 1915, xxiii, 317, and earlier papers.

Method.

Preparation of the Sample.—3 cc. of whole blood, plasma, or serum are run slowly (a slow stream of drops) from a pipette into about 75 cc. of a mixture of redistilled alcohol and ether (3 parts alcohol, 1 part ether) in a 100 cc. graduated flask. The contents of the flask should be kept in motion during the process so that there is no clumping of the precipitated material. The contents of the flask are raised to boiling by immersion in a water bath (with constant shaking to avoid superheating), cooled to room temperature, filled to the mark with alcohol-ether, mixed, and filtered. The filtered liquid if placed in a tightly stoppered bottle in the dark will keep unchanged for a considerable time so that if it is not convenient to complete the determination at once, the sample may be carried to the above stage and left till a more suitable time.

By running the blood slowly into the large quantity of alcohol-ether, as above, the protein material is precipitated in finely divided form and under these conditions the short heating combined with the great excess of solvent is adequate for complete extraction of serum or plasma. The extraction, while not quite so complete in the case of whole blood, is believed to be better (because of the higher values obtained) than that obtained by any other method in use at the present time.

Determination.—10 cc. of the alcohol-ether extract are measured into a small flat-bottomed beaker and evaporated *just* to dryness on a water bath or electric stove. Any heating after dryness is reached produces a brownish color which passes into the chloroform and renders the subsequent determination difficult or impossible. The cholesterol is extracted[5] from the dry residue by boiling out three or four times with successive small portions of chloroform and decanting into a 10 cc. glass-stoppered, graduated cylinder which has previously been calibrated. The combined extracts after cooling (5 cc. or less) are then made up to 5 cc. The solution should be colorless but not necessarily clear, since the slight turbidity clears up on adding the reagents.

[5] In order to get an adequate extraction with the small amounts of chloroform used, an excess (3 or 4 cc.) should be added each time and the mixture allowed to boil down to half its volume or less, before decanting.

5 cc. of a standard cholesterol solution in chloroform[6] (contain
ing 0.5 mg. of cholesterol) are measured into a similar 10 cc
cylinder.

To each of the solutions are added 2 cc. of acetic anhydrid
and 0.1 cc. of concentrated sulfuric acid, the solutions mixed b;
inverting several times, then set away in the dark for 15 minutes
after which they are transferred to the cups of the colorimete
(Duboscq) and compared as usual, setting the standard at 15 mm
The cement of the colorimeter cups must, of course, not be solubl
in chloroform; plaster of Paris has been found satisfactory, o
even ordinary glue if the cups are not used for any other purpose
In using the colorimeter in this determination the window scree
described by Folin and Denis[7] has been found valuable. Th
error of the above method when carried out with ordinary care i
4 to 5 per cent. If greater accuracy is desired it may be obtained
at the expense of more material and time, by using 50 cc. of th
alcohol-ether extract, evaporating as above, extracting witl
larger quantities of chloroform, making the extracts to 25 cc.
and taking an aliquot of 5 cc. for the determination.

RESULTS.

Cholesterol added to alcohol-ether extracts of blood was re
covered quantitatively. Parallel determinations were carried ou
with blood plasma by the Autenrieth-Funk method (ether ex
traction) and by the adaptation described above. The result
are summarized in the table.

The results obtained by the new procedure are always highe
(7 to 30 per cent, average 20 per cent) than by the Autenrieth
Funk method. Also the colors produced in the chloroform ex
tract by the new procedure are almost invariably of the sam
tint as the standard, in marked contrast to those by the Auten
rieth-Funk procedure, which almost always have the yellowish o
brownish tinge mentioned above. In many cases this discolora

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[6] It is convenient to make the cholesterol standard in two strength
(a) the stock solution containing 0.2 gm. of cholesterol (Kahlbaum) i
200 cc. chloroform; and (b) the standard solution for use, made by dilutin
10 cc. of the above to 100 cc. with chloroform. 5 cc. of this latter solutio
will contain 0.5 mg.

[7] Folin, O., and Denis, W., J. Biol. Chem., 1914, xviii, 263.

Plasma samples.	Cholesterol per 100 cc.	
	Autenrieth-Funk method.	New procedure.
	mg.	*mg.*
Dog I	180	210
" II	120	135
" III	141	147
" IV	100	130
Pig I	94	117
" II	80	104
Sheep	75	95
Rabbit	31	42
Human (carcinoma)	216	240
" " (syphilis)	112	140
" " (nephritis)	200	250

tion was so strong as to make the readings doubtful. Possible explanations for the higher results by the new procedure are: (1) the previously mentioned difficulty of extracting cholesterol from a strongly alkaline solution; and (2) changes in the cholesterol produced by the action of the strong alkali during the long digestion at high temperature in contact with the oxygen of the air. Autenrieth and Funk state, however, that further extraction produces no higher results, which would indicate that the second was the more probable explanation. That cholesterol is not entirely stable under conditions similar to the above has been pointed out by Lifschütz.[8]

⁸ Lifschütz, J., Z. physiol. Chem., 1906–07, 1, 436.

MASS ACTION IN THE ACTIVATION OF UNFERTILIZED STARFISH EGGS BY BUTYRIC ACID.

By RALPH S. LILLIE.

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INTRODUCTORY.

In experiments performed at Woods Hole in the summer of 1914 I found that unfertilized starfish eggs, after treatment for several minutes with weak solutions of butyric acid in sea water (N/260), not only formed fertilization membranes on return to normal sea water, but cleaved more or less regularly and developed into freely swimming larvæ (blastulæ and gastrulæ).[1] In order to induce favorable development by this means the exposure must be several times longer than that normally allowed for artificial membrane formation; otherwise the treatment is the same; the eggs are simply returned from the acid-containing solution to normal sea water after a definite time in the solution, and receive no fur-ther treatment. In favorable experiments the great majority (80 to 90 per cent) of eggs thus activated formed larvæ; in similar experiments performed during the past summer the proportion of active and well developed larvæ has frequently reached 95 per cent or more. This treatment thus forms a highly favorable parthenogenetic method with starfish eggs; it does not, however, succeed with sea urchin eggs, which require a supplementary treatment (*e.g.*, with hypertonic sea water) to induce complete activation. In the experiments of Aug. 31 and Sept. 1, 1914, the optimal exposures varied from 6 to 10 minutes; briefer exposures of 2 to 5 minutes caused typical membrane formation, but cleavage was imperfect or delayed, and development usually stopped short of the swimming stage; with still briefer exposures of 1 minute

[1] Lillie, R. S., *Biol. Bull.*, 1915, xxviii, 260.

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or less the eggs merely formed membranes and broke down without development. The effect of underexposure is thus to induce an activation which is imperfect or partial; in order to complete this activation all that is needed is to prolong the exposure sufficiently; the same result may be gained by treating the underexposed eggs a second time, after an interval in sea water, with the same fatty acid solution for an appropriate period.[2] There is thus a well defined optimum duration of exposure to the activating solution; exposures of less than this optimum are insufficient for complete activation; exposures prolonged a few minutes above the optimum injure the eggs, and development ceases early; with still longer exposures the eggs fail to cleave or even to form membranes.

The effects of higher temperatures on unfertilized starfish eggs (i.e., of exposure to sea water at 30–35°) vary in an essentially similar manner with the duration of exposure; at each temperature a certain minimal exposure is required to induce membrane formation (at 32° about 2 minutes); one several times longer causes complete activation (about 8 minutes at 32°); while with still longer exposures (more than 12 minutes at 32°) the eggs fail to develop. The precise time of exposure required to produce each of these effects decreases rapidly as the temperature rises, being approximately halved by a rise of 1°.[3] Varying the time of exposure to warm sea water has thus the same physiological effect as similarly varying the exposure to a fatty acid solution,—a fact indicating that with both agents the essential condition underlying activation is the same.

The question arises as to the nature of this condition. It is to be assumed that the primary effect of exposure to the high temperature or to the fatty acid solution is to initiate some chemical process in the egg—probably an interaction between two substances already present in the protoplasm; this process has a critical or determinative relation to the normal sequence of physical and chemical changes constituting development. The above time relations indicate that in order to bring the egg into a con-

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[2] Lillie, *Biol. Bull.*, 1915, xxviii, 291. After-treatment with hypertonic sea water, cyanide, or warm sea water (32–34°) may also be used to complete the activation.

[3] Lillie, *Biol. Bull.*, 1915, xxviii, 278 and 279, Tables IX and X.

dition in which it is capable of continuing automatically its development to an advanced stage, this critical interaction must proceed until a definite quantity of reaction product has been formed; it is to be presumed that the reaction product is then present in its appropriate situation at a certain definite concentration. If this concentration is not reached activation is only partial, and only a few of the early steps of development are carried out, as seen in the formation of the fertilization membrane, and the execution of some cleavages of less than normal rate; if it is exceeded the egg is also rendered incapable of prolonged development, though for a different reason. That the failure of development after membrane formation by brief exposure to the activating condition is due simply to an insufficient formation of this reaction product, is indicated by the fact that a second treatment, either with fatty acid or high temperature, completes the activation and enables the egg to form larvæ. No such treatment will restore the power of development to eggs that have been overexposed to either agent.[4]

It seems clear that some single process, comparatively simple in its nature, is started by the activating agent; this process is progressive and requires time, and in order to induce normal development it must proceed to a certain stage and then be arrested. The nature of the chemical factor in the activation process cannot be inferred with definiteness from the above data; some light, however, appears to be thrown on the condition under which the critical combination takes place. When unfertilized eggs are brought into sea water at 30°, the activation process is initiated and continues progressively as long as the egg is kept at this temperature, reaching its completion in about 30 minutes; at 32° the same stage of activation is reached in 7 to 8 minutes, at 34° in 3 minutes, and at 36° in 1 minute. The activation process thus exhibits a high temperature coefficient (Q_{10} = 200 to 400); this coefficient is similar in its order to that of the decrease of viscosity or degelation of colloidal systems of gelatin and water under the influence of rising temperature.[5] This fact suggests that the

[4] It is possible that the effects of overexposure may be reversible under other conditions, but this has not yet been determined.

[5] Compare von Schroeder, P., Z. physik. Chem., 1903, xlv, 75; Levites, S. J., Z. Chem. u. Ind. Kolloide, 1907-08, ii, 211; also the discussion in the paper cited above, Lillie, Biol. Bull., 1915, xxviii, 294.

direct effect of the high temperature upon the egg is to cause some
change in the colloidal consistency of certain regions or structures,
involving decrease of viscosity or degelation; this latter change
permits the combination of constituents which in the resting egg
are kept apart through their inability to diffuse. Under such
conditions the rate of combination will be determined by the rate
of diffusion (the reciprocal of viscosity), and hence will show the
same temperature coefficient as the decrease of viscosity. The
specific nature of the chemical combination involved is, however,
not indicated by experiments with high temperature alone.

The fact that in the activation of eggs by butyric acid the same
relations are found as in heat activation between time of exposure
and physiological effect produced, suggests that the first chemical
change in the activation process is a combination in which an acid
takes part. It is evident, however, that any acid concerned in
normal fertilization or heat activation cannot be a fatty acid, but
must be one that diffuses less readily through the protoplasmic
system; the possibility suggests itself that it may be an hydroxy-
acid like lactic acid,—which moreover is known to be produced
by stimulation in many protoplasmic systems. If the primary
chemical event in activation is a chemical union between an acid
and some other component of the egg system, and if this acid
may be replaced by the rapidly diffusing butyric acid, the acti-
vating effect of butyric acid should be influenced by tempera-
ture and concentration in the manner characteristic of chemical
reactions; *i.e.*, the rate of the activation process should be in-
creased in a definite proportion by a given rise of temperature,
or by a given increase in the concentration of the acid.

The question is whether butyric acid activates the starfish egg
by combining chemically with some protoplasmic constituent, *i. e.*,
by forming a compound like an ester, or by altering some physical
condition, such as the state of the colloids at the egg surface, thus
indirectly enabling another and critical combination to take
place. The low concentration and brief exposure required for the
action indicate that a direct alteration of the colloids is not the
primary effect produced by the acid. On the other hand, the
differences observed by Loeb[6] in *Strongylocentrotus* eggs with fatty

6 Loeb, J., *Biochem. Z.*, 1909, xv, 254; Artificial Parthenogenesis and
Fertilization, Chicago, 1913, 133.

ıcids, hydroxy-acids, and mineral acids, indicate that all acids are
ılike as regards the possession of activating power; the rate of
ɔenetration is the chief factor determining action in any particu-
ar case. Loeb and Hagedoorn also found that the least effective
luration of exposure to butyric acid solution was halved by rais-
ng the temperature from 10° to 20°,[7] also indicating that the acid
ıcts by chemical combination. If this is the case, the minimal
:ime of exposure ought also, according to the mass action law,
;o be halved (at constant temperature) by doubling the concen-
ration of the acid; but a proportionality of this kind does not
ıppear to exist in *Strongylocentrotus* eggs.[8] The conditions in
:his egg are, however, more complex than in the starfish egg;
ıcid causes only partial activation (membrane formation) which
ıas to be completed by an appropriate after-treatment; *e.g.*, with
ıypertonic sea water. Bases as well as acids may also cause
nembrane formation in sea urchin eggs;[9] the membrane-forming
·ffect would thus appear in this egg to have no direct relation
o the chemical nature of the agent employed. In the starfish
·gg the conditions differ in two essential respects: first, the acti-
ʳation following the proper exposure to butyric acid is complete;
.nd second, this effect cannot be produced by a typical base like
.mmonia. The complete lack of any activation effect with bases,
.s contrasted with the completeness of the effect produced by
.cid, seems especially significant. This negative action is illus-
ʳated by the following experiment. On June 14 the unfer-
ɨlized eggs from several starfish were exposed to solutions of
.mmonium hydroxide of the following concentrations: 0.0005 N,
· .001 N, 0.0015 N, 0.002 N. Van't Hoff's artificial sea water was
sed to avoid the disturbing effects of carbonates. Eggs were
·laced in each of these solutions at about 20° during the early
.ıaturation period, between the breakdown of the germinal vesicle
.nd the separation of the first polar body, and were returned to
ʟormal sea water at intervals of 1, 2, 3, 4, 6, 8, 10, 12, 15, 18, 21,
ʹ5, 30, 35, and 42 minutes. The results were uniformly negative;
ʀı no case was there membrane formation or cleavage; all eggs

ʳ Loeb, Artificial Parthenogenesis and Fertilization, Chicago, 1913, 146.
ʼ Loeb, Artificial Parthenogenesis and Fertilization, Chicago, 1913, 135.
ʼ Loeb, Artificial Parthenogenesis and Fertilization, Chicago, 1913, 147;
I . Exp. Zool., 1912, xiii, 577.

died without signs of development, as in the untreated control.
The fertilized control eggs showed normal development. The
range of concentrations and exposures employed is amply suffi-
cient to prove the ineffectiveness of this base as activating agent.
In *Strongylocentrotus* eggs, on the other hand, solutions of am-
monia and other weak bases in about N/1675 concentration (0.3
cc. 0.1 N base plus 50 cc. sea water) were found by Loeb to be
highly effective membrane-forming agents. It seems clear, there-
fore, that in the *Asterias* egg bases and acids differ entirely in
their relation to the activation process; and since both butyric
acid and ammonia are lipoid-soluble compounds and penetrate
the egg readily, it is to be inferred that the contrast in their
physiological effects is determined by the difference in their gen-
eral chemical properties; *i.e.*, that acids act by combining chemi-
cally with some protoplasmic constituent and not by their
physical action on the colloids.

Experiments on the relative rates of action of butyric acid so-
lutions of constant concentration on starfish eggs at different
temperatures have not yet been tried. Experiments at constant
temperature with different concentrations of butyric acid indicate
clearly that a direct relation exists between concentration and
rate of action. With eggs of uniform condition and a range of
concentrations of 0.00075 N to 0.006 N it was found that the
time of exposure required for activation varied inversely with the
concentration, showing a close approach to the linear relation
required by the law of mass action. This comes out most clearly
in a series of experiments performed during early June (Table
III), at which time the condition of the starfish eggs at Woods
Hole is most uniform and favorable. The same relation was also
found later in the summer (July and August), although the eggs
were then less regular in their behavior and responded more rap-
idly to the action of the acid; *i.e.*, the effective exposures for a
given concentration were shorter.

EXPERIMENTAL.

In the following experiments the eggs were placed in the solu-
tion of butyric acid during the early maturation period, before
the separation of the first polar body, and after the time interval
indicated they were returned to normal sea water. The condition

of the eggs and the proportion developing to larval stages we
afterwards determined. In the earlier series the solutions
butyric acid were made by adding the 0.1 N solution to sea wat
In the later experiments the butyric acid solutions were made
van't Hoff's artificial sea water, to avoid the disturbing influen
of neutralization.

The essential results of this treatment are the same with
effective concentrations of acid; the briefest exposures indu
membrane formation, which may be normal, but is followed
imperfect cleavage and early breakdown; as exposure is pi
longed more and more above this minimum the proportion
cleaving and favorably developing eggs increases progressive
reaching a maximum with a certain time of exposure which
shorter the more concentrated the solution; with further increa
of exposure the proportion of eggs reaching larval stages aga
declines; cleavage becomes slower and development ceas
sooner; eventually even fertilization membranes fail to form.
partial or imperfect activation is readily induced; with any giv
concentration of acid a relatively wide range of exposures m:
thus be effective for inducing membrane formation or the first f
cleavages; but in order to insure the development of all norm
eggs to larval stages the time of exposure to the solution must
definite. It is noteworthy that the rate of cleavage is fast
and the cleavage itself more regular the nearer the exposure a
proaches the optimum.[10] In the case of any solution of butyi
acid, the manner in which the degree of activation varies wi
varying times of exposure may be represented by a curve with t
percentage of eggs forming larvæ as ordinates, and times of e
posure as abscissæ; this curve has an approximately symmetric
form;[11] its range as measured along the abscissæ is shorter t
higher the concentration. In the case of heat activation t
curve relating the duration of warming (e.g., at 32°) to the pi
portion of eggs forming larvæ has a similar form.[12] The tir
range occupied by this curve decreases with rising temperatui

[10] Lillie, *Biol. Bull.*, 1915, xxviii, 283, Table XII.

[11] Lillie, *Biol. Bull.*, 1915, xxviii, 282.

[12] See Lillie, *Biol. Bull.*, 1915, xxviii, 279, Table X, for a summary of t
data.

just as that of the curve of acid activation decreases with rising concentration of butyric acid.

Table I gives a summary of all of last summer's experiments in which eggs were exposed for varying times to solutions of butyric acid in sea water. The 0.1 N butyric acid was added to sea water as indicated (0.001 N means 1 cc. of 0.1 N butyric acid plus 99 cc. of sea water). The carbonates of sea water partly neutralize the acid added, and the 0.001 N and 0.002 N solutions are for this reason ineffective; the other solutions all cause membrane formation and development with sufficient times of exposure. The duration ranges from 1 to 25 minutes; in each series eggs were transferred from the acid solutions to sea water at regular intervals,—of 1 minute with the briefer exposures; of 2 to 3 minutes with the longer ones. The table gives in each series the range of exposures used and the optimum duration of exposure, together with the approximate proportion of eggs forming larvæ at this exposure. In some cases this optimum was not reached.

The optimum exposure thus decreases progressively with increase in the concentration of butyric acid. With the six effective solutions the respective optima are: 0.003 N, doubtful (more than 30 minutes); 0.004 N, about 30 minutes; 0.005 N, 12 to 15 minutes; 0.006 N, 8 to 12 minutes; 0.007 N, about 6 minutes; 0.008 N, about 5 minutes. The relation between time of exposure and concentration is not constant in this series, as seen in the difference between 0.004 N and 0.005 N; but the results with artificial sea water indicate that this divergence is due simply to the removal of part of the butyric acid by neutralization; if a correction is applied for this effect an approximately constant inverse proportionality is seen. Acid added to 0.002 N concentration is completely, and to 0.003 N in greater part neutralized, as regards its physiological effect on the eggs; if we assume that with the remaining solutions the only effective acid is that present in excess of 0.003 N, the following results are obtained (Table II), which agree closely with those obtained a few days later with artificial carbonate-free sea water (Table III). The product of corrected concentrations and effective times of exposure should, according to the law of mass action, be a constant. Table II indicates that this is approximately the case; these results should be compared with Table IV, which gives the results of similar experiments with artificial sea water.

TABLE I.

Date.	Concentration.	Range of exposures.	Results.
	N	min.	
May 31	0.001	3-12	No membranes; eggs die without change.
" 31	0.002	3-12	" " " " "
" 31	0.003	3-12	All eggs form membranes; with 12 min. exposure 10-20 per cent form larvæ; optimum not reached.
June 2	0.003	1-15	15-20 per cent form larvæ with 15 min. exposure; optimum not reached.
May 31	0.004	3-12	30-40 " " " " 12 " " "
June 2	0.004	1-15	35-40 " " " " 15 " " "
" 4	0.004	3-25	65-75 " " " " 25 " " probably ± 30 min.,
" 2	0.005	1-15	Optimum at 12 min., with ± 85-90 per cent larvæ.
" 4	0.005	3-25.	" " 14-17 " " ± 90 " " At 20 min., 70-80 per cent; at 25 min., 30-40 per cent.
" 4	0.006	3-25	Optimum at 10-14 min., with 90-90 per cent larvæ. At 17 min., 25-30 per cent.
" 5	0.006	1-20	" " 7-8 " " 70-80 " " " 10 " 50-60 " " At 17 min., 5-10 per cent.
" 5	0.007	1-20	No larvæ with 1-2 min.; optimum at 6 min., 90-95 per cent larvæ; at 10 min., 30-35 per cent; none at 14 min.
" 5	0.008	1-20	At 2 min. 1-2 per cent form larvæ; optimum at 5 min. (> 90 per cent); at 7 min., 35-45 per cent; none at 10 min.

During the week of June 7 to June 13 similar experiments were carried out with solutions of butyric acid in van't Hoff's artificial sea water (100 volumes 0.5 M NaCl + 7.8 vol. 0.5 M MgCl$_2$ + 3.8 vol. 0.5 M MgSO$_4$ + 2.2 vol. 0.5 M KCl + 2 vol. 0.5 M CaCl$_2$) using the concentrations 0.0005 N, 0.00075 N, 0.001 N, 0.0015 N, 0.002 N, 0.0025 N, 0.003 N, and 0.004 N. All these solutions cause membrane formation and activation; with the 0.0005 N solution exposures of less than 4 minutes proved insufficient for membrane formation, and the longest exposures (42 minutes) caused only 4 to 5 per cent of the eggs to develop to larvæ; with all the other solutions the optimum exposure was reached, at which 90 per cent or more of the eggs formed larvæ. The effect of varying the duration of exposure was the same in all cases;

TABLE II.

Concentration of acid as added.	Corrected concentration of acid (C).	Optimum time of exposure (T).	Product (CXTX10³).
N	N	min.	
0.001	0		
0.002	0		
0.003	?	? >30	
0.004	0.001	±30	±30
0.005	0.002	12–17	24–34
0.006	0.003	8–12	24–36
0.007	0.004	.±6	24
0.008	0.005	±5	25

the briefest effective exposures induce typical membrane formation, followed by irregular change of form and breakdown; with longer exposures the proportion and rate of development increase progressively up to a well defined optimum, after which there is a second decline.

The results of these experiments are indicated in Table III. The optimum duration of exposure with each solution, and the proportion of eggs forming larvæ are given as before. The solution of butyric acid in van't Hoff's sea water was added to a small volume of eggs; at intervals eggs were transferred by pipettes to normal sea water.

Throughout these experiments the control eggs showed uniformly normal behavior, practically all undergoing maturation

TABLE III.

Date.	Concentration.	Range of exposures.	Results.
	N	m.n.	
June 13	0.0005	1-42	Only 4-5 per cent form larvæ with 42 min. exposure; optimum not reached.
" 13	0.00075	1-42	80-90 per cent form larvæ with 42 min. exposure; this exposure is probably about the optimum; no longer exposures were made.
" 7	0.001	3-25	Longest exposure, 25 min.. gave only 1-2 per cent larvæ; optimum not reached.
" 8	0.001	3-20	Only 1-2 per cent larvæ with 20 min. exposure; optimum not reached.
" 13	0.001	1-42	Optimum at 35 min. with about 95 per cent larvæ; at 42 min. rather less favorable, 80-90 per cent.
" 10	0.0015	1-20	40-50 per cent larvæ with 20 min. exposure; optimum not reached.
" 13	0.0015	1-42	Optimum at 20-25 min. with ± 95 per cent larvæ; at 30 min., 20-25 per cent.
" 7	0.002	3-25	" " ±14 " " 80-90 " " ±65-70 " "
" 10	0.002	1-20	" " 15 " " 70-80 " " 65-75 " "
" 11	0.0025	1-20	" " 12 " " >95 " " ±90, at 17 min., 35-40 per cent.
" 7	0.003	3-25	Optimum at 7-10 min. with 80-90 per cent larvæ with each exposure at 7, 8, and 10 min.; at 14 min., ± 40-50 per cent.
" 11	0.003	1-20	Optimum at 10 min., with > 95 per cent larvæ: 8 and 12 min. also favorable, with 80-90 per cent; at 14 min., 30-40 per cent.
" 7	0.004	3-25	Optimum at 6-7 min., with ± 85-90 per cent: 8 min. is only slightly less favorable, with ± 75-80 per cent; at 10-12 min., 10-15 per cent.

and developing normally after fertilization; the above results
may therefore be regarded as typical. Later in the summer the
condition of the eggs was more variable, and the optimum time of
exposure to a given solution proved decidedly shorter,—a change
of condition due probably to a combination of factors (higher
temperature, approaching end of breeding season, etc.). Experi—
ments carried out later in the season are described in Table V.

In Table IV I have summarized the results of all the experiments
in which a large majority of eggs (80 per cent or more) formed
swimming larvæ with the optimal times of exposure. Experi—
ments performed with different solutions upon the same lot of
eggs are placed together under the same date. Usually some

TABLE IV.

Date.	Concentration (C).	Optimum time of exposure (T).	Product (C×T×10⁷).
		min.	
June 7	0.002	±14	±28
" 7	0.003	7–10	21–30 (average 25.5)
" 7	0.004	6–7	24–28 (" 26)
" 10	0.002	±15	±30
" 11	0.0025	12–14	30–35 (average 32.5)
" 11	0.003	8–12; 10 best	24–36 (" 30)
" 13	0.00075	±42	±31.5
" 13	0.001	±35	±35
" 13	0.0015	20 and 25	30–37 5 (average 34

variation in the condition of the eggs is observed from day t
day, but at this time of year such changes are comparativel
slight; later they may become considerable, as Table V shows.

These results show that with solutions ranging from 0.00075
to 0.004 N the product of concentration and optimal time
exposure is an approximate constant for each solution, with a
average value of about 30. In other words, the velocity of th-
activation process—the change within the egg enabling it to con
tinue development to a larval stage—is proportional to the con-
centration of the butyric acid. This fact furnishes strong evi
dence that the acid acts by combining chemically with som
constituent of the egg system, and that the presence of a definit

quantity of reaction product, which must not be exceeded, is the prerequisite for complete activation.[13]

Other experiments carried out later in the summer yielded a similar result, but the value of the CT product was in all cases lower than the above; i.e., the eggs become more sensitive to the activating influence of the acid; the experimental results also show greater variability. The results are summarized in Table V, which includes all those experiments in which a large proportion (50 per cent or more) of mature eggs formed swimming larvæ. The experiments were all performed between July 28 and Aug. 9, except the first two (June 28 and July 1); in these the eggs appear to show an intermediate condition.

From July 28 to Aug. 3 the relation between duration of effective exposure and concentration of acid shows some irregular fluctuation, but on the whole it remains fairly constant; the average value of the product CT during this period is about 12; this is less than half the value observed in early June (about 30); from Aug. 4 the product is again higher (average about 19) and resembles that of the two series of June 28 and July 1. The condition of the starfish eggs at this time of year, near the end of the breeding season, is variable, and perhaps a greater constancy is not to be expected. The reciprocal of the product CT may be regarded as a measure of the sensitivity of the eggs to the acti-

[13] These results might conceivably be interpreted as indicating merely that the essential requirement for activation is the entrance of a certain minimal quantity of acid into the egg, and that the rate of entrance is a direct function of the concentration gradient between the medium and the cell interior. If, however, the simple rate of entrance is the essential factor determining the above relation between concentration and rate of activation, we should expect that the effective times of exposure would be only slightly affected by temperature. In the above cited experiments of Loeb and Hagedoorn with Strongylocentrotus eggs the time required for membrane formation was found to be halved by raising the temperature from 10° to 20°. I have not yet investigated the influence of temperature on the rate of acid activation in the starfish egg. In general it seems probable, from the rapidity with which lipoid-soluble substances enter cells, that the time required for simple entrance in the above experiments is negligible in comparison with the time required to produce the physiological effect within the egg. If the rate of this activation effect is proportional to the concentration of the activating agent, and shows the chemical temperature coefficient, the presumption seems fair that the agent acts by combining chemically with some cell constituent.

vating influence of the acid; *i.e.*, as a coefficient of sensitivit;
What determines these variations of sensitivity is uncertain; du
ing the warmer part of the summer CT showed a lower value tha
earlier or later; *i.e.*, the eggs responded then most quickly to tl
acid; the change on Aug. 4 seems to correspond with a change :
the weather which at that date turned decidedly cooler.   I hav
not as yet made definite experiments on the influence of ten

TABLE V.

Date.	Concentration (C).	Range of exposures.	Optimum (T).		Result of exposure next above optimum.		Product (CXTX1C
		min.	*min.*	*per cent*	*min.*	*per cent*	
June 28	0.003	1-17	6	90	At 7 and 8, 70-80		18.0
July 1	0.003	1-17	7-8	80-90	" 10, 20-30		21-24
" 28	0.005	0.5-4	2	±95	" 2.5, 80-90; at 3, ±50		10.0
" 28	0.006	0.5-4	1.5	90-95	" 2, ±50		9.0
" 29	0.0035	1-10	3	70-80	" 4, 50-60		10 5
" 29	0.0045	1-10	2	65-70	" 3, 10-15		9.0
" 30	0.0035	1-6	2.5-3	85-90	" 3.5, 65-75		8.8-10
" 30	0.0045	1-6	1.5-2	70-85	" 2.5, 25-35		6.8-9.(
" 31	0.003	1-9	2-2.5	85-90	" 3, ±50		6.0-7.:
" 31	0.004	0.5-4.5	1	70-80	" 1.5, 35-45		4.0
Aug. 1	0.003	0.5-5.5	3	80-90	" 3.5, ±50		9 0
" 1	0.004	0.5-5	1.5	40-50	" 2, 2-3		6.0
" 3	0.003	0.5-5	4-4.5	80-90	" 5, 75-85		12.0-13
" 3	0.004	0.5-5	2.5-3	85-90	" 3.5, 65-75		10-12.
" 4	0.002	1-12	9-10	80-90	" 12, 75-85		18-20
" 7	0.002	1-14	9	85-90	" 10, 65-75		18.0
" 7	0.0025	1-14	7-9	80-90	" 10, 60-70		17.5-22.
" 9	0.0015	2-22	12 14	55-65	" 16, 15-20		18.0-21.

perature on this property of the eggs.[14]   Internal factors, such a
variations in the physiological conditions of the animals at dii

[14] I have found, however, that the presence of various chemical sut
stances in the medium may greatly alter the time of exposure required fc
activation, either by high temperature or fatty acid solution.   This is illui
trated by the following experiment.   On July 1 eggs were exposed to 0.0C
N butyric acid dissolved (*a*) in unaltered van't Hoff's solution, and (*b* an
c) in van't Hoff's solution containing respectively 3 and 4 volumes pe
cent ethyl alcohol.   The optimum time of exposure in the absence of alce
hol (*a*) was 7 to 8 minutes (70 to 80 per cent larva); in both the alcoho.
containing solutions the optimum was about 3 minutes (80 to 90 per cen
larvæ), and somewhat shorter in *a* than in *b*; 5 minutes was well past th

ferent periods of the breeding season, are probably more important. Of chief interest is the fact that at the height of the season, early June, when the eggs are most abundant and most uniform in behavior, CT had a value of approximately 30 and showed relatively slight variation. This condition probably represents the norm in these eggs.

SUMMARY.

1 - Unfertilized starfish eggs may be caused to develop into larvæ by a sufficiently prolonged exposure to weak solutions of butyric acid in sea water or van't Hoff's solution. No supplementary or "corrective" treatment is required.

2 - In order to produce complete activation the eggs must be exposed to the solution for a certain minimal time which is a function of the concentration of acid. Exposures briefer than this optimum cause partial activation,—membrane formation followed by imperfect cleavage and breakdown; activation may be completed by a second exposure to the same solution. Exposures longer than the optimum injure the eggs and interfere with activation.

3 - Activation is a progressive process which under uniform conditions of temperature and physiological state of the eggs appears to proceed at an approximately uniform rate. This rate is closely proportional to the concentration of butyric acid, within a range of 0.0005 N to 0.006 N.

4 - The most consistent interpretation of these facts seems to be that the acid activates the egg by combining chemically with some egg constituent until a certain quantity of a definite reaction product is formed. The time required to produce this critical quantity will, in accordance with the mass action law, be inversely proportional to the concentration of acid.

optimum, while brief exposures (2 minutes) produced a considerable number of larvæ (in b 10 to 15 per cent; in c 20 to 30 per cent), in contrast to a where an exposure of 4 minutes was required to produce 5 to 10 per cent larvæ. The whole activation process thus proceeds from two to three times more rapidly in the presence of alcohol than in its absence. Alcohol alone has no activating effect; it merely promotes or facilitates the action of the fatty acid,—sensitizes the egg, as it were. Alcohol similarly shortens the effective time of exposure to warm sea water. The basis of such effects is uncertain as yet; but they show that the rate at which the activation process proceeds depends on certain modifiable physicochemical conditions in the egg system. I hope to consider this subject at greater length in a separate paper.

THE PRESENCE OF EPINEPHRIN IN HUMAN FETAL ADRENALS.

By JULIAN HERMAN LEWIS.

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A number of investigators have found epinephrin in considerable amounts in the adrenals from fetuses of different animals, by both chemical and physiological methods. Some of the same investigators and others have been unable to detect it in human fetal adrenals. Moore and Purinton[1] analyzed human fetal adrenals by the FeCl₃ and blood pressure methods with negative results. Svehla,[2] using the blood pressure method, did not find epinephrin in the human fetus, but found it in the adrenals of the dog fetus. Langlois and Rehns[3] were able to show pressor substances in sheep fetal adrenals. Fenger[4] found large amounts of epinephrin in the adrenals of beef fetuses, and McCord[5] obtained physiological reactions in the sixth week of gestation in cattle. Cannata[6] did not examine the adrenals, but he tested the serum of healthy new-born infants for epinephrin. His results were negative.

It is difficult to understand the differences in the physiology of the fetuses of man and the lower animals that would explain why one should possess epinephrin and the other not. Suspecting that the disparity in the findings of previous investigators might lie in the use of faulty methods, a series of analyses of human adrenals was begun. Recently Folin, Cannon, and Denis[7] have

[1] Moore, B., and Purinton, C. O., *Am. J. Physiol.*, 1900-01, iv, 57.
[2] Svehla, K., *Arch. exp. Path. u. Pharm.*, 1900, xliii, 321.
[3] Langlois, J. P., and Rehns, J., *Compt. rend. Soc. biol.*, 1899, li, 146.
[4] Fenger, F., *J. Biol. Chem.*, 1912, xi, 489.
[5] McCord, C. P., *J. Biol. Chem.*, 1915, xxiii, 435.
[6] Cannata, S., *Pediatra*, 1915, xxiii, 244.
[7] Folin, O., Cannon, W. B., and Denis, W., *J. Biol. Chem.*, 1912-13, xiii, 477.

described a chemical method for determining epinephrin in tissues, which is both simple and delicate. A reagent is used which consists of a mixture of phosphoric acid and sodium tungstate. The reagent gives a blue color with solutions containing epinephrin. Uric acid also gives the same color, but this is, for the same amount, three times more intense and is therefore used as a standard in titrating tissues for epinephrin. This test seemed admirably adapted for the problem undertaken. Several investigators have used it in other work and have reported it to be satisfactory.

The method of proceeding was to obtain the fetus as fresh as possible. The adrenals were taken out, dissected free of adventitious tissue, weighed, and ground up in 0.1 N HCl and H$_2$O with sand. The mixture was boiled with the addition of sodium acetate according to the directions of Folin and Denis. After being made up to a standard volume, the extract was tested with the phosphotungstate reagent. The blue color produced was compared with that produced by 1 mg. of uric acid. The results are seen in Table I.

TABLE I.

"Epinephrin" Content of Fetal Adrenals as Determined by the Folin-Denis Colorimetric Method.

Age of fetus.	Weight of two adrenals.	Amount of "epinephrin" in both glands.
mos.	*gm.*	*gm.*
3	0.304	0.00058
3	0.222	0.00029
3	0.464	0.00033
5	1.825	0.0004
7	1.515	0.00033
Full term, still birth.	2.34	0.00066
Full term, still birth.		0.00166

If these determinations represent the actual amounts of epinephrin in the adrenals, the amounts are large enough to be detected by other methods. Therefore another series of analyses was begun with the blood pressure method, Seidell's manganese dioxide test,[8] the FeCl$_3$ test, and the AuCl$_3$ test.

[8] Seidell, A., *J. Biol. Chem.*, 1913, xv, 197.

All these tests were negative when made on a series of human fetal adrenals ranging from 3 months to full term. Boiled NaCl extracts of these adrenals constantly gave a marked fall in blood pressure and never an undoubted pressor effect. We are aware that extremely small amounts of epinephrin cause a fall in blood pressure instead of a rise. The injection of an extract of fetal liver or kidney will produce a similar depressor action, which is ascribed to the reaction with foreign proteins or the products of their cleavage. And possibly some of it is due to the action of choline. It would be impossible to determine if an additional fall of blood pressure due to small amounts of epinephrin was superimposed on the fall produced by simple tissue extracts.

It occurred to us that probably the reason for the finding by the Folin-Denis method of amounts of epinephrin in human fetal adrenals that could not be detected with other methods is that fetal tissues contain a relatively large amount of uric acid. Uric acid has been found in considerable quantities in the kidneys of the new-born, leading to the formation of the so called uric acid infarcts. Whether or not other fetal or infantile tissues contain much uric acid cannot be determined from the literature, because no reference can be found to analyses of such tissues for uric acid.

A 7 months fetus was obtained and the kidneys were removed. It was then ground up and analyzed by the Krüger-Solomon method for uric acid. 100 mg. were found. These results are striking because corresponding amounts of uric acid have not been found in normal adult human tissues. The adrenals from the same fetus and four other fetuses were ground up and extracted with 0.1 N HCl; the extract was analyzed physiologically for epinephrin and chemically for uric acid. The physiological test was negative and the chemical analysis showed 0.005 gm. of uric acid.

Uric acid accumulates in human tissues because they do not contain enzymes that can destroy uric acid. This is not true of most other mammals. In order to determine whether the Folin-Denis test agreed with the physiological test for epinephrin in tissues that do not contain such large quantities of uric acid, two sets of analyses were made of fetal beef adrenals. 53 gm. of adrenals were obtained from beef fetuses, unselected as to size. These were ground up and extracted with 0.1 N HCl. This extract

was titrated for epinephrin by the blood pressure method, with
Parke-Davis adrenalin 1:3,200 as a standard. By this method
0.045 gm. of epinephrin was found. The Folin-Denis test gave
0.06 gm. Another lot of 193.5 gm. was obtained and analyzed
by the same methods. The physiological test gave 0.087 gm.
of epinephrin, and the Folin-Denis test gave 0.12 gm. Analyses
of this lot by the Krüger-Solomon method for uric acid gave
0.003 gm., the presence of which may be accounted for by the
fact that uricase is not formed until late in the development
(Mendel and Mitchell[9]).

The most delicate test for epinephrin is the method in which an
intestine or uterus strip contracting in oxygenated Ringer's solu-
tion is used. This method is reported to detect epinephrin in a
dilution of 1:1,000,000. As a final experiment for the purpose
of finding epinephrin in human fetal adrenals, the glands from a
6 months fetus were obtained 18 hours after death. The fetus
had been kept on ice during this time. A boiled extract was
made of the ground up adrenals in 6 cc. of Ringer's solution. A
guinea pig was killed instantaneously by a blow on the head, and
a piece of the small intestine about 3 cm. long was tied at one end
to an L-shaped glass rod and to a sensitive lever at the other
end, and then immersed in a tube containing warm Ringer's solu-
tion through which bubbled a slow stream of oxygen. The tube
containing the strip was put into a large tank of water which was
kept uniformly at 39°C. When contractions were well estab-
lished the effects of adrenalin and the extract of the fetal adrenals
were determined. These solutions were kept in the same bath with
the strip and were introduced into the tube through an opening in
the bottom. Adrenalin stopped the contractions and inhibited the
tone promptly while the extract had no effect whatever.

The adrenals from two full term fetuses were next obtained and
extracts of the ground up glands were made with Ringer's solution.
One of the fetuses had lived a few seconds, and the other half an
hour after birth. The extract of the former was tested with a
strip of guinea pig intestine contracting in vitro under the same
conditions described in the preceding experiment, and the extract

[9] Mendel, L. B., and Mitchell, P. H., Am. J. Physiol., 1907-08, xx, 9-7.

of the latter tested with a strip of rabbit uterus and intestine. The tone and contractions of the two intestinal strips were inhibited, while the tone of the uterine strip was increased, indicating the presence of epinephrin in the glands of both fetuses. These experiments were sufficiently controlled by comparing the effect of adrenalin.

These last two experiments are the nearest to an actual demonstration of the presence of epinephrin in human fetal adrenals that we have obtained, yet they are not free from objections because such sensitive objects as intestinal and uterine strips contracting *in vitro* are apt to be not specific in their response to substances acting on them. Stewart[10] emphasizes that such tests should be controlled with other biological tests.

There is reason to suspect that epinephrin is contained in the human fetal adrenals. These glands in the fetus are strikingly large, in proportion to the size of the other organs, when compared with the adult adrenals, and there is no doubt that their size has some relation to their functional activity. As far as we know, the needs of the human fetus differ in no respect from those of the lower animals, and no reason suggests itself for the absence of epinephrin in one and its presence in the other. A possible explanation for the difficulty in demonstrating it in human fetal adrenals lies in the fact that a normal human fetus is rarely obtained. Spontaneous and surgical abortions are usually the result of some abnormal condition of the mother. Facts concerning prenatal pathology are meager and we know nothing of the influence of these conditions on the fetus. Usually the fetus is subjected to prolonged asphyxia during delivery which may influence the adrenals. It may also be that oxidative enzymes are more active in the fetus than in the adult, and that post-mortem destruction of epinephrin is more prompt.

The Folin-Denis test is undoubtedly the best colorimetric test that has been devised for the detection of epinephrin, but, like all its predecessors, has its limitations. The experiments recorded in this paper make clear one of its most serious disadvantages. Just as it cannot be used to titrate epinephrin in fetal tissues, it ob-

[10] Stewart, G. N., *J. Exp. Med.*, 1912, xv, 547.

viously cannot be used with adult tissues from gouty individuals, because of their high content of retained uric acid.[11] Neither can it give accurate results in the assay of tissues from birds, because of their characteristic uric acid metabolism.

SUMMARY.

1. Epinephrin could not be found in human fetal adrenals by the use of the blood pressure method and chemical tests.

2. A suggestion of the presence of epinephrin was found in the adrenals of two full term fetuses by the use of strips of uterus and intestines contracting in warm oxygenated Ringer's solution. The extract of the adrenals of a 6 months fetus tested with a contracting intestinal strip was entirely negative.

3. The Folin-Denis test cannot be used to determine epinephrin in fetal tissues, because of their high content of uric acid.

[11] Fine, M. S., and Chace, A. F., *J. Biol. Chem.*, 1915, xxi, 371.

CELL PENETRATION BY ACIDS.

By W. J. CROZIER.

CONTENTS.

INTRODUCTION.

A study of the chemoreceptors of the earthworm *Eisenia foetida*,
begun at the suggestion of Professor G. H. Parker, resulted in the
accumulation of data which could not be analyzed in terms of
current ideas relating to cell permeability and its rôle in stimula-
tion. At the same time it led to certain views regarding the prob-
lem of irritability; namely, that this question may best be studied
with the sense organs of animals of moderate structural complexity,
rather than in simple[1] protozoa or in relatively non-specialized
cells. In a ceptor cell the processes of irritability stand forth with
special clearness, because less obscured by the course of other
activities than is probably the case with the majority of tissue
cells; irritability is so predominantly the function of the ceptor

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[1] Dobell (1914, page 181) states: "It is safe to prophesy that when the
known facts have been doubled or trebled, the ironical statement . . . .
that 'the Protozoa are the simplest organisms in which to study the great
problems of biology' will disappear from biological literature."

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that it may here be studied in a reasonably pure condition.[2] Organs of chemical sense seem particularly well adapted for such experiments. Moderate morphological complication is a necessary restriction for technical reasons, since it is only with animals of not too high an organization that quantitative methods of experiment involving the measurement of responses may successfully be used.

Part of the study referred to dealt with stimulation by acids, and the facts observed in this connection were responsible for the present work on the penetration of cells by acids.

Previous to the recent paper of Harvey (1914), cell penetrability for acids had not been studied by any direct and approximately quantitative method, largely because, as Harvey remarks, cells containing indicators appropriate for this work have seldom been observed.[3] He was able to utilize the pigmented gonidial filaments of a holothurian, *Stichopus ananus* Jaeger, and measured the time required for the testis of this animal to change color when placed in 0.01 N solutions of a variety of acids. The results of his experiments with acids of this concentration led Harvey to the view that probably lipoid solubility, or capillary activity, and affinity for proteins of the cell surface were both factors in determining the particular penetration speed observed with a given acid, but that there was no agreement between penetration speed and ionization.

During the summer of 1913 I found that the blue pigment of a nudibranch, *Chromodoris zebra* Heilprin, gave an extract with dependable qualities as an indicator, and in a brief note (Crozier, 1914) I described some properties of the substance. It was also found that acids would change the color of the indicator within the cell.

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[2] I have since observed that a similar idea has been developed by Lucas (1909, particularly page 328): "The physiology of the cell must begin, not with that maid-of-all-work, the unicellular organism, but with such cells as are as nearly as possible unifunctional." "The only practical method for the study of any function will be to investigate that function first in some cell in which it appears in a highly elaborated state."

[3] The statement is often made that indicators are of rare occurrence in animals, but I have discovered such materials during the last 3 years in such widely separated forms as: a balanoglossid, a common Bermudian and West Indian tunicate, and an annelid, in addition to the one herein described.

Harvey's list of acids, though embracing a number of substances, was studied at only one concentration, and previous work on stimulation by acids had convinced me that series purporting to indicate the relative physiological activity of acids were not likely to be of general value when constructed on such a basis. This expectation has been completely justified (Crozier, 1915). It is with this problem, the penetration rate of acids as a function of concentration, and the interpretation of stimulation by acids, that the present account is concerned. The conclusions to which I have come regarding permeability to acids are in a way similar to those of Harvey, aside from the part assigned to ionization, but the method of analysis is different and based upon more complete data.[4]

The Indicator.

The only characteristics of the *Chromodoris* indicator which are important here are (a) its mode of occurrence and (b) the influence of various conditions upon the magnitude and rapidity of its color changes. Some hints have been obtained as to the chemical nature of the material and its significance for the so called warning coloration of the animal, but these will be described in another place.

For example, this indicator gives positive results with nine tests for alkaloids, but otherwise gives no reaction with protein precipitants; it appears to be intimately connected with the animal's spicy odor and general immunity from enemies. The pigment appears to bear some relation to the blue material described by von Zeynek (1901, 1902) from the fish *Crenilabrus*. From the fact that the blue substance is decolorized on reduction and may be restored to its original hue by H_2O_2, some writers would doubtless regard it as a respiratory pigment.

(α) *Occurrence.*—The position of the pigment in the cell cannot be made out in sections, because it dissolves. Observations are therefore restricted to teased cells and tissues manipulated in a compressorium. Such observation shows that several forms of the pigment are present. Along the border of the mantle it can be seen that by far most of the material is situated at the base of the epithelial cells, in the form of sac-like bodies of variable

[4] The control of permeability to acids by salts, and other phases of this problem, are also being studied.

size and (apparently changeable) shape. But blue granules occur quite near to the outer surface, and the small spherical bodies which occur in the mucus on the animal's surface are strongly tinged with blue.

The coloration of *Chromodoris* depends upon the blue pigment and a yellow one. Along the border line between blue and yellow patches, cells can be made out which contain both yellow and blue pigment, like the doubly pigmented erythrophores of *Ostracion* described by Ballowitz (1913); but such cells are infrequent.

(b) *Color Changes.*—The yellow pigment is not soluble in water and cannot be extracted by aqueous alcohol, aqueous acetone, or pyridine. It is soluble in xylol, full strength alcohol, acetone, and other fat solvents. Its globules stain with dyes that color fat.
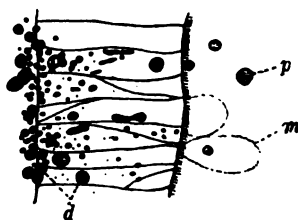
Fig. 1. From a camera sketch of a compressed preparation of the edge of the mantle. d = dense blue bodies; p = pale spherical masses; m = mucus. $\times$ 125.

The blue bodies indicated below the basement membrane were located in near-by cells of the epithelium, the outlines of which are not shown at this focus.

The blue substance, which is soluble in water, is the only indicator involved. Pyridine, a weak base, apparently extracts it in the least changed condition, the solution being blue or blue-green. Alcoholic or aqueous extracts are at first blue with a reddish fluorescence, but subsequently turn purple, with a strong blue fluorescence. As an indicator this pigment behaves somewhat like litmus, though its tints are more pronounced. Solutions of the pigment in fresh water or in 4 per cent solutions of formalin in either fresh water or sea water, and alcoholic extracts or aqueous solutions of the residue from an evaporated pyridine extract, give sensibly the same end-point when a few drops are added to 10 cc. of test solutions. The hydrogen-ion concentration for the

transition blue → pink, determined by phosphate and acetate mixtures, is the same, $p_H = 5.6 \pm$; this holds for solutions containing sea-water salts (NaCl + KCl + CaCl$_2$, at $\frac{5}{8}$ M total concentration), therefore corresponding presumably to the condition in the cell. 0.001 N solutions *in vitro* of the eighteen acids I have used effect the blue → pink transformation instantly. Dilute solutions containing the proteins of *Chromodoris* may give apparent end-points as low as $p_H = 4.7$.[5]
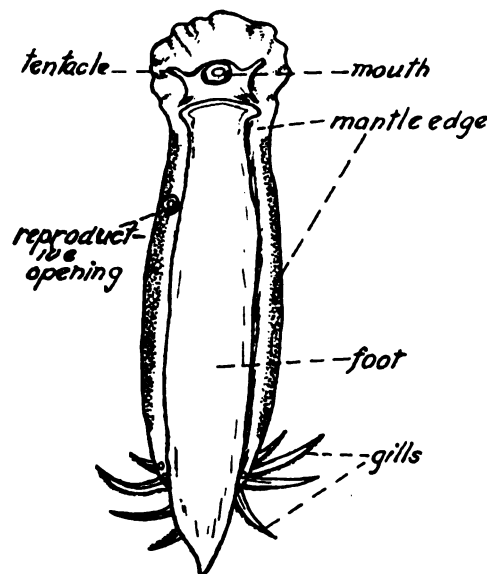
FIG. 2. *Chromodoris zebra*, diagrammatic ventral view; stippled areas show regions from which pieces were cut for the study of acid penetration.

Method.

Pieces were cut from the lateral mantle edge of *Chromodoris* shown by Fig. 2. The animals were in every case freshly collected and in good condition, though I cannot agree with Smallwood (1910, page 140) that these animals do not well survive laboratory confinement. The pieces excised were about 10 mm.

[5] Compare Harvey (1913).

long by 5 to 6 mm. broad at the widest place, but tapering to a point at either end, and usually 2 to 3 mm. thick along the line of cutting, thinning rapidly to less than 0.5 mm. at the extreme outer edge. The penetrability of the different regions of

TABLE I.

Comparative Penetrability of Different Parts of Chromodoris to 0.01 N Acetic Acid. Time of Visible Penetration in Minutes.

	Penetration.
	min.
Rhinophores..	1.0
Tentacles...	1.5
Buccal mantle..	4.0
Gills..	4.0
Ventral surface of marginal mantle...................	75
Dorsal " " " "	>120
Foot, edge...	100
Buccal margin of pharynx............................	35
Inner wall " "	90

TABLE II.

Penetration of Pieces Cut from the Midlateral Mantle of Individuals (Chromo———doris) of Different Sizes. The Pieces Were All of the Same Size, Two or Three from Each Individual. 0.1 N HCl.

Animal.		Penetration. (27°C.)		
No.	Length.			
	cm.	*min.*	*min.*	*min.*
1	2.5	1.00	1.25	1.00
2	3.7	1.75	1.50	
3	3.9	1.75	2.00	2.25
4	. 5.5	1.00	1.25	1.20
5	6.2	3.00	3.25	
6	6.4	3.50	3.00	3.25
7	7.8	3.75	4.00	
8	10.4	2.75	3.50	3.25

an animal varies considerably (Table I), and for animals of di**
ferent sizes the speed with which acids produce color change **i-**
pieces cut from corresponding locations is somewhat related to t**h-**
length of the individual, as indicated in Table II, smaller spec**i**

mens tending to show greater penetrability. Consistent results were, however, easily obtained when these size and location factors were made uniform, and especially when the under surface of the mantle was employed.[6] Animals 8.5 to 10 cm. long were used.

The density of the blue coloration varies widely in different individuals, though the general scheme (Table I) is consistent for any one animal, as far as the area we are considering is concerned (Fig. 2). Material was therefore selected from animals showing a smooth blue pigmentation of the under surface of the mantle.

The source of error involved at this point lies less in the different metabolic conditions with which the several degrees of pigmentation may be correlated, and in the fact that the concentration of the indicator may affect its rate of color change by acids, than in one's inability to judge

TABLE III.

Penetration of Pieces of the Lateral Mantle by 0.01 N HCl (27°C.).

Coloration.		Penetration.				
		1	2	3	4	5
		min.	*min.*	*min.*	*min.*	*min.*
A	Purplish blue....	5.5	5.0	6.7	6.9	7.3
B	Blue............	8.0	6.9	7.2	9.0	5.4
C	Spotted.........	8.9	9.6	7.5	8.1	7.3

accurately as to the exact point at which the pink hue becomes evident. *Chromodoris* shows four rather well defined conditions as regards the character of the blue mantle pigmentation; these may be termed the albino (see Smallwood, 1910, page 141), the blue, the purplish blue, and the spotted. The spotted condition is one in which the blue coloration is confined to circular dots less than 1 mm. in diameter, which are separated by distances usually of several mm., the intervening surface being pure white or slightly tinged with blue. These color varieties are not very clear-cut, the blue showing occasionally a spotting of denser color, but for convenience I have used them in Table III to show the variation in penetration time observed with the different modes of coloration.

Chromodoris is an hermaphrodite, and animals of the size we are dealing with reproduce at all times of the year; therefore sex

[6] Part of the pigment on the dorsal surface seems to be in a different physical or chemical condition, such that it is not easily dissolved out or changed in color.

and position in the reproductive cycle are of no consequence in the present connection.

The pieces to be tested were removed by a clean cut with sharp scissors while being held gently with forceps. They were then allowed to remain in sea water for about 10 minutes. As one result of the cutting there occurred a considerable secretion of slime. The pieces also contracted, in part through loss of the water which gives the tissue turgescence, and the edges of the cut surface began to roll inward over the plane of the cut. Immediately before transfer to the acid the pieces were washed in fresh sea water and carefully rolled between layers of clean filter paper; this wiping, which removed the slime and adherent sea water, was necessary to give uniform results, but control tests showed that treatments of this sort from one to five times in succession, allowing one-half minute in sea water between successive applications of the filter paper, did not affect the penetration time of the acids.

Each experiment consisted in immersing a piece of the *Chromodoris* tissue in a vessel[7] of thin colorless glass holding about 25 cc. of acid. The acid solutions were made up in recently caught rain water. Contact with the acid led to a further production of slime, the mucin of which was promptly coagulated. That the thin layer so formed did not affect the penetration of the acid was proved by experiments in which the film of mucin was carefully lifted off with forceps; this did not accelerate penetration to any measurable degree. At intervals of about a minute the solutions were stirred very gently. The volume of the pieces was 0.5 cc. $\pm$, and it was found that this ratio of 1: 50 between the volumes of tissue and solution was sufficient to preclude a decrease in acidity of the fluid during the longest time over which an experiment extended (120 minutes). The glass vessel was placed upon a slip of white porcelain, and the color changes were viewed in diffuse light from a northeast window.

Even after long practice it is necessary to have comparison standards, if a color change—even so distinct a one as that here employed—is to be judged promptly. For this purpose I have used two fresh pieces of tissue cut from the same animal and of the same size as the piece to be tested,

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[7] Small crystallizing dishes were used.

one of the fragments (blue) being kept in normal sea water and the other in sea water recently made acid (pink) either by 0.01 N HCl or by some of the acid being tested. These controls were placed on the porcelain surface beside the piece whose color change was being timed.

The time elapsing between immersion in acid and the assumption of the pink color was taken with a stop-watch. In order to avoid color fatigue, the pieces of tissue were not viewed continuously but inspected at intervals of about a quarter of a minute. The point of transition from blue to pink could be determined with an accuracy indicated by a maximum error of 10 to 15 seconds. *It is important to note that the color change did not first*

TABLE IV.

Data from One Experiment Comparing the Penetrability of Living and of Dead Cells (Experiment 56.1). *

Acid.	Control.	Dead cells.
	min.	*min.*
HCl, 0.1 N	2.2	1.2
	2.1	0.9
H₂SO₄, 0.05 N	5.0	2.5
	2.6	1.1
	2.5	1.0
Acetic, 0.02 N	20.0	9.0
	17.0	6.0

* The control pieces were cut from positions on the opposite side of the animal in the same relative location as the corresponding cells killed with 4 per cent formalin.

occur along the edge of the cut and from there progress laterally, but appeared evenly and with reasonable sharpness over the whole surface of the pieces studied. The blue pigment is more dense at the dorsal and ventral surfaces of the mantle than in the deeper tissues, but it could be seen that a faint pink hue was produced on the cut surface; this did not influence the development of the color transition on the ventral side.

A point of importance is the relation of the penetration rates of acids in the case of cells killed previous to immersion in acid. Killing by heat causes the coagulation of the pigment and the assumption of a green color; now, alkalies induce this reaction *in vitro*, the p_H value for the change being about 7.6 (at 27°), and

the effect of heat upon the indicator within the cell is to be ascribed to the increased alkalinity brought about by the thermal coagulation of proteins (Quagliariello, 1912). Cells killed by formalin do not give constant results, but the indication (Table IV) is (1) that cells so killed are more easily penetrated than those not previously killed, and (2) that the increased penetrability is not such as to make the cells readily permeable for all acids, though it amounts to about twice that of cells treated with acid while living.

Penetration Speed and Concentration.

Proceeding in the manner indicated by the previous section, the time of penetrations from a number of concentrations was determined for eighteen acids. The results are summarized in Table VII. Ten or more observations were made at each dilution. The general concordance of the separate measurements, which naturally is better at higher concentrations, may be illustrated by the records in Tables V and VI.

As I have elsewhere remarked (Crozier, 1915), the series of penetration rates from 0.01 N solutions shows a noteworthy correspondence with Harvey's (1914) measurements at the same dilution. The reasonable closeness of this agreement (Table VIII), considering that two widely separated animals were employed, and that in one case a portion of the integument and in the other an internal protected organ (testis) furnished the cells experimented upon, makes it logical to assume that a similar order of acid penetration is generally valid.

TABLE V.

Penetration of Chromodoris Tissue by 0.01 N HCl (Experiment 10.13).[*]

No.	Penetration.	No.	Penetration.
	min.		*m'n.*
1	9.0	6	8.0
2	7.0	7	8.25
3	8.0	8	6.9
4	7.5	9	7.25
5	5.5	10	8.5

Mean...................................... 7.6 ± 0.78

[*] A second series, 3 months later, gave 7.5 ± 0.50.

TABLE VI.

Experiments with Oxalic, Monochloroacetic, and Acetic Acids.

No.	Penetration.		
	Oxalic, 0.002 N.	Monochloroacetic, 0.005 N.	Acetic, 0.01 N.
	min.	min.	min.
1	18.0	18.3	57
2	19.5	17.0	90
3	20.6	13.0	70
4	17.5	14.2	67
5	19.6	17.5	85
6	16.3	12.5	60
7	19.5	16.5	86
8	18.0	20.0	78
9	21.0	11.3	80
10	25.0	16.0	76
Mean	19.5 ±1.6	15.6 ±2.4	75 ±9.1

TABLE VII.

Acid.	Penetration time in minutes (27 °C.).																		
Normal dilution.	10	15	20	50	75	100	125	150	200	250	300	325	350	400	500	650	700	750	1,000
Hydrochloric	2.9			5.8	7.0	7.6			9.5						12.5			16.3	20.0
Sulfuric	2.7		3.2	5.9	7.5	8.5		10.5		13.5					17.0			24.7	
Oxalic	4.0			6.0	8.0	8.8				13.5					19.5	25.0			
Nitric	3.6			5.8		8.4				14.5			17.7		22.0		30.0		
Formic	1.75				4.0	4.5		9.2	13.0	15.0					24.5				
Salicylic				1.8	2.1	3.6	6.4	8.0	11.6	16.3	18.5	32.0			56.5				
Valeric (iso-)				1.2	1.5	1.9		6.0	12.0		26.0			55.0	70.0				
Monochloroacetic	5.6			7.4		10.0				15.5		24.0							
Malonic			4.7			10.0			15.2	22.5	24.0	36.0							
Benzoic						9.7			14.8			29.7							
Lactic	4.6			6.0	7.9	8.6	11.5			21.3	32.5								
Tartaric	5.5			8.8		13.5					32.0	60.0							
Malic	5.9		6.1			14.5					37.5	67.0							
Citric	7.0					16.0		26.0		40.0									
Succinic		6.9				16.5					45.0								
Butyric	7.6			12.6	15.0	19.0		30.0		50.0									
Propionic	7.1					30.0		55.0											
Acetic	11.9			19.5	30.5	75.0													

TABLE VIII.

Cell Penetration from 0.01 N Solutions.

Acid.	Chromodoris, mantle edge (27°).	Stichopus, testis (Harvey).
	min.	*min.*
Valeric (iso-)	1.9	2–4
Salicylic*	3.6	0.25
Formic	4.5	2–4
Hydrochloric	7.6	}
Nitric	8.4	
Sulfuric	8.5	9–11
Lactic	8.6	
Oxalic	8.8	12–15
Benzoic*	9.7	0.25
Monochloroacetic*	10.0	2–4
Malonic	10.0	} 30
Tartaric	13.5	
Malic	14.5	} 40
Citric	16.0	
Succinic	16.5	—
Butyric	19.0	}
Propionic	30.0	45–60
Acetic	75.0	

* The acids not corresponding in the two series are marked with an asterisk.

The curves of Fig. 3 show the relation between normality dilution (*i.e.*, liters containing one equivalent) and average penetration rate. They bring out features of the penetration process which measurements at a single dilution (Harvey, 1914) leave obscure. Series showing the relative penetrating power of acids vary with the concentration chosen (Crozier, 1915) and it follows that existing correlations of the mode of penetration for different acids with lipoid solubility, toxicity, and the like, must be revised. The form of the dilution curve as a whole must be used to determine the position of an acid in the penetration series.[8] On this basis the acids studied may be separated into two groups, an end also attained, though with less accuracy, by consideration of the limiting concentrations beyond which no color change is effected inside the cell.

[8] A consideration of this kind applies likewise to studies on the effects of acid solutions of equal hydrogen-ion content.
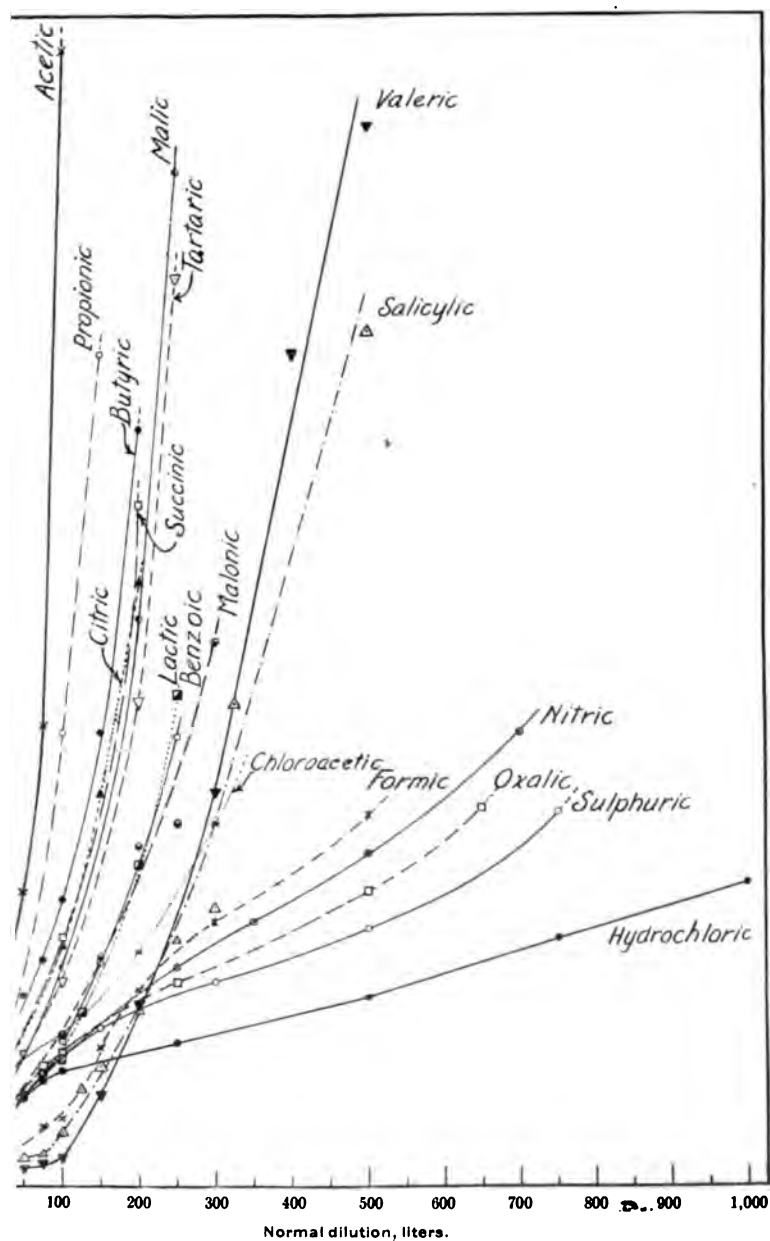
FIG. 3. The relation between dilution and time of penetration.

Outward diffusion of pigment occurs in acid solutions but this diffusion does not bear any constant relation to the time occupied by the internal color change, being variable and depending in part upon the degree to which the piece of tissue is contracted or passively stretched. The use of this outflow of pigment as an index of permeability changes is therefore not free from an objection which may be urged against much recent work on quantitative aspects of the permeability problem; namely, that cell pigment may be squeezed out in purely mechanical fashion, or that mechanical stresses may control the speed and extent of pigment loss. In general, with solutions giving rapid penetration, the spontaneous outward movement of pigment occurred after the color change within the cell; with lower concentrations, before an internal condition of acidity had been made visible. Moreover, as will be shown subsequently, it is possible experimentally to increase or decrease the penetration time of a given acid solution, and in certain of these cases the state of permeability as revealed by pigment diffusion is exactly the reverse of that shown by the speed of acid penetration.

The order of decreasing efficiency in penetration for the eighteen acids studied is approximately as indicated in Table IX, where position in the series is compared with ionization strength.[9]

At first sight there is little precise concordance between penetration speed and ionization, but from the tangle of curves the following sets of chemically related acids may be taken as probably significant.

1. The acids of high ionization are obviously set apart from the others by the character of their dilution curves, while as a whole the most weakly ionized acids are at the other end of the series; and I take the relative position of the members of these two groups to indicate that ionization is the primary determinant of cell penetrating power, but that some other factor[10] controlling entrance into the cell is also operative. On the one hand the ionization of HCl, H_2SO_4, $(COOH)_2$, and HNO_3[11] is so great that the second factor has relatively free sway in fixing their individual position, while, on the other hand, the ionization of the fatty acids is so weak that here also another factor determines the order of penetration, excepting that of formic acid, which is ioni-

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[9] For the first $H^{\cdot}$.

[10] It is hardly probable, and certainly not necessary, that this second factor should be the same for different acids.

[11] Borowikow (1913) noted the order HCl > H_2SO_4 > HNO_3 > $(COOH)_2$ for the acceleration of plant growth.

cally stronger than the others (compare its different type of curve).

2. Among the weak fatty acids the order of penetration is: *valeric* > *butyric* > *propionic* > *acetic*; some clue to the mechanism of the process is clearly afforded by the fact that this is precisely the order of their action upon the surface tension of water and of their solubility in xylol (Harvey, 1914, page 948).[12]

TABLE IX.

Order of Penetration Compared with Ionization.

Acid.	Ionisation constant.
Hydrochloric	(100.0)
Sulfuric	(100.0)
Oxalic	3.8
Nitric	(100.0)
Formic	0.0214
Salicylic	0.1020
Valeric (iso-)	0.0017
Monochloroacetic	0.155
Malonic	0.163
Benzoic	0.0060
Lactic	0.0138
Tartaric	0.100
Malic	0.0395
Citric	0.0870
Succinic	0.00665
Butyric	0.00153
Propionic	0.00137
Acetic	0 00187

3. Monochloroacetic acid is more highly ionized than acetic, and penetrates more easily.

4. With the saturated dibasic acids the order of penetration is: oxalic > malonic > succinic, which parallels exactly that of their ionization; and here again oxalic, the acid of much greater ioniza-

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[12] This would appear to relate the activity of these acids to their lipoid solubility, a view favored by Harvey (1914) and, for other acids, by Vernon (1913), though on insufficient evidence. Their exact relation to the fatty substances of *Chromodoris* cells has not yet been determined.

tion than the other two, is set apart from them by the character of its curve.

5. Hydroxy substitutions, which result in increased ionization, give three clear-cut instances of increased penetrating ability likewise accompanying this change: (a) *lactic > propionic*, (b) *tartaric > malic > succinic*, (c) *salicylic > benzoic*.

There consequently seems no escape from the conclusion that ionization is a primary factor in determining cell permeability for acids;[13] if sufficiently great, the degree of dissociation practically controls the situation, and when chemically related acids are considered it fixes their relative penetrating power with precision; if sufficiently low, as in the fatty acid series aside from formic, some factor allied to capillary activity is dominant—though at the same time it is evident that the constitution of the other acids studied is likewise of importance in relation to the make-up of the cell surface and substance. The general result of these experiments is to indicate that while some material other than lipoids, which may be identified as protein,[14] is primarily concerned in permeability (compare Loeb, 1912[b], and Osterhout, 1913, page 141), yet the cell surface must be regarded as complex and totally misrepresented by some current simple theories of permeability, which explain fairly well the conditions of penetration for single classes of substances (compare Loeb, 1912[a], 1914, page 443; Lillie, 1913; Höber, 1914, page 349).

Comparisons with Stimulation.

Series expressing the relative activity of acids as they affect a number of processes might be compared with the data presented in this paper, were it not for the fact that in many cases such a comparison, at least on the quantitative side, would involve the possibility of a serious fallacy. This is particularly true of figures purporting to give the relative toxicity of acids, and the fallacy lies here: the times required for the same concentration of different substances to produce a given effect are commonly as-

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[13] This is also indicated by the results of Dale and Mines (1911) regarding the contraction of muscle in acids.

[14] Shown also by experiments, to be described elsewhere, on the penetration of *Chromodoris* tissues by acid-salt combinations.

sumed to be proportional to the concentrations of these substances which will produce another result within some fixed time; on the contrary, the dilution-*versus*-effect curves for various substances may be of entirely different character (compare Fig. 3), and little correspondence of such acid series could reasonably be expected; moreover, the mechanism of absorption of substances from *very* dilute solutions may be quite different from that at high concentrations (compare, for example, the sense of smell in vertebrates).

(a) I wish particularly to attempt an explanation of sensory stimulation by acids, on the basis of the present data, but a more general case of stimulation may first be considered—that of induced membrane formation in the sea urchin egg (see Loeb, 1913, page 133). The order of acid effectiveness in leading to membrane formation is given, in general, as:

Monobasic fatty acids > dibasic acids > mineral acids,

which is just the reverse of that obtained in considering cell penetration. Within these groups of acids we find stated (Loeb, 1913, page 133) the following approximate order of efficiency (in 0.5 M NaCl solution).

TABLE X.

Orders of Efficiency in Membrane Production. [*]

Butyric > *propionic* > *acetic* > formic.
Propionic > lactic.
Benzoic > *butyric*.
Oxalic, tartaric > *citric* > *succinic*.
HNO₃ > HCl(> H₂SO₄).

[*] The italicized instances agree with the penetration series.

Loeb (1913, page 139) comments upon the apparent disagreement of these facts with the dissociation theory, but points out that it is only the mass of acid which has actually entered the egg that is to be considered, and that the magnitude of this inwardly diffusing mass depends upon the constitution of the acid. But it will be noted that it is only in certain particulars that the membrane and penetration series agree, and that the relative position of the fatty, dibasic, and mineral acids, of formic acid, and of hydroxy substitution in the case of lactic acid, is reversed. Harvey's series for acid penetration, by agreeing fairly well with

that obtained for the *Chromodoris* tissue at the same concentra-
tion, strongly suggests the validity of my acid series for tissue
cells generally, and we must conclude either that the acids lead-
ing to membrane formation act merely upon the surface of the
egg, which is chemically different from the substance of the inte-
rior,[15] or else that the permeability of egg protoplasm for acids is
qualitatively different from that of tissue cells. Both conclusions
may be correct; but the fact of membrane formation shows that
the surface of the mature egg is in its physicochemical make-up
different from that of the generality of cells, and the short time in
which very dilute solutions of certain acids will bring about mem-
brane formation, as compared with their speed of diffusion through
protoplasm, makes it seem certain that the parthenogenetic power
of acids involves only a reaction with substances at the surface
of the egg, not the egg protoplasm as a whole. This view is not
incompatible with Kite's (1913) conclusion that for certain sub-
stances the permeability of egg surface and cytoplasm are the
same. As far as the evidence for acids is concerned, we must
regard the egg surface not as a simple thing, but as quite com-
plex and as containing not only elements which are similar to
those of tissue cells,[16] but also others or else the former in different
proportions.

(b) Turning now to the matter of sense organ stimulation by
acids, it may first be pointed out that the order of permeability
to acids for different regions of the body of *Chromodoris* (see
Table I)—*viz.*, anterior tentacles, rhinophores > gills > buccal
mantle > posterior mantle edge > lateral mantle (under surface)
> edge of foot > dorsal body surface—appears to be also the order
of the sensitivity of these parts to acids. This statement is made
on the basis of work on the behavior of *Chromodoris* by Dr. L. B.
Arey,[17] and of independent experiments of my own.

Tests have been made upon marine animals, namely *Holothuria
surinamensis*, *Stichopus moebii*, and *Ptychodera* sp., and upon

[15] The fact that isolated egg fragments can form membranes is no objec-
tion to this interpretation.

[16] Compare the instances in which the penetration series holds (Table
X) for parthenogenesis, and the fact that cell-permeability and membrane-
formation series are the same for alkalies (Harvey, 1911; Loeb, 1912[c]).

[17] I am indebted to Dr. Arey for permission to include certain of his
observations in advance of publication.

earthworms, which show that, as with human taste, the limiting dilutions beyond which various acids are non-active in stimulation follow this order: fatty acids < dibasic < mineral acids, which agrees in a general way with the penetration series.

For more detailed comparison, data obtained from experiments with the worm *Eisenia foetida* may be considered. These experiments were carried out in the following way:[18]

Each worm (*w*, Fig. 4) was placed upon a low ridge (*r–r*) dividing into two compartments (*a, b*) a trough constructed of paraffin walls upon a ground glass plate (*p*); compartment *a* contained an acid solution, *b*, water once distilled, to a depth of about 1 cm., each compartment holding 50 cc. of fluid. The anterior end of the worm being immersed in the acid solution, it is stimulated by the acid, and the worm withdraws into the water. The time occupied by this response (from the instant of immersion until that of
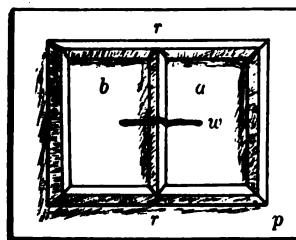
Fig. 4. View of apparatus for stimulation experiments with earthworms.

complete retraction, measured with a stop-watch) is used as an estimate of the amplitude of the reaction due to stimulation by the particular solution,—in other words, as an index of the intensity of stimulation. The figures given are the means of twenty-five tests at each concentration for each acid.

The time of response so obtained is not strictly a reaction time, as some have termed it;[19] but it is the time required for the performance of a definite act as the result of stimulation,—quite a different thing, and a quantity which we may reasonably hold

[18] Details of this method, particularly of the dozen or more recognized sources of error which surround it, and the means taken to overcome and estimate these complicating factors—such as the size of the animals, their previous history, the proportion of their length immersed, etc.—cannot be described here. Posterior stimulation was also studied.

[19] Parker and Metcalf (1906), Hurwitz (1910), Shohl (1914).

to be proportional to stimulus intensity.[20]   I mean by a stimulus the alteration produced in a receptor,[21] not the concentration (density) of the stimulating agent.

In comparing these curves of response (Fig. 5) to several dilutions of a number of acids, the following are the outstanding
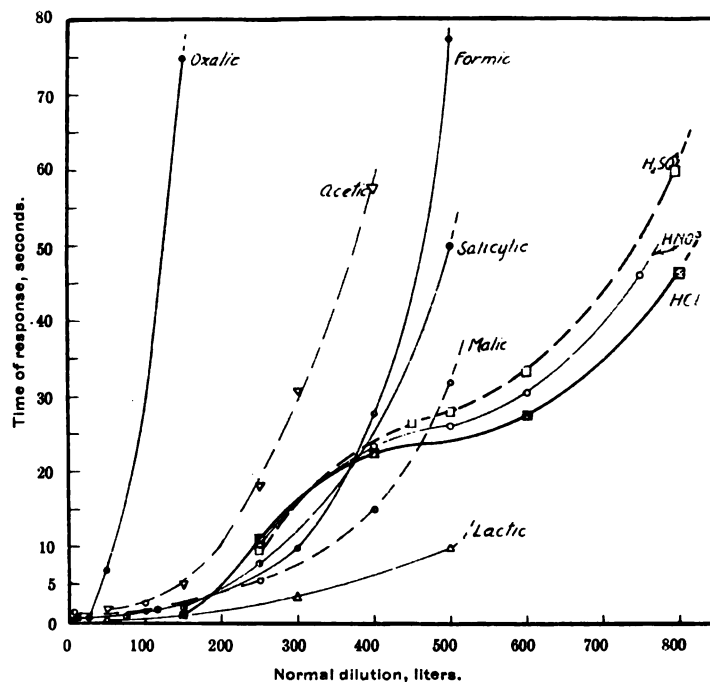
Fig. 5.   Time of response to dilutions of nine acids, for the earthworm (see text).   No measurable responses were obtained above the dilutions shown.

points and the only ones which will be considered here: (1) Different types of curves are obtained (a) with strong mineral acids,

[20] Or to the difference between this intensity and a factor expressing the tendency of the worms to progress normally in a forward direction.   There are further complexities when the stimulation of the tail end is considered.

[21] In the present case, rather the integral of the partial stimulations experienced by the individual ceptors, according to whatever scheme this integration is accomplished in the nervous system of the animal.   Except for the prostomium, there is good morphological ground for the opinion that this scheme may be fairly simple.

and (b) with weak organic acids.[22] (2) The strong acids follow the order of their ionization, but this at such high dilutions that the correspondence probably has little real reference to dissociation. (3) The weak acids fail to stimulate at lower dilutions than do the strong acids. (4) The weak acids are more stimulating in proportion to their ionization than would be predicted if this were the only factor involved.

The points numbered (1), (3), and (4) find their exact counterparts in the penetration series, but the time quantities involved make it obvious that the ceptor surface only is concerned in stimulation. That there is no detailed correspondence in the position of acids in the two series is not surprising, since this worm lives commonly in manure piles, and its chemoreceptors are therefore probably specialized in a peculiar way.

Points (3) and (4) have, of course, long been recognized in taste stimulation (human), and indeed appear universally when chemoreceptors are considered. Various explanations have been suggested to account for these facts, such as the possible greater ionization of the part of the acid at the cell surface, etc.; but it seems to me that explanations of this type cannot hold. The parallelisms between the stimulation series and the penetration series show unmistakably that chemical stimulation involves the solution of the stimulant in the surface of the ceptor cell; and from such solutions or combinations the requisite electrochemical effects can readily be derived (compare Beutner, 1912).

Further evidence in this direction is afforded by the fact that, if the worms are successively stimulated at short intervals (30 seconds), data are obtained such as are shown in Fig. 6. Two aspects of these curves are significant: (1) the temporary lengthening of the time of response after several stimulations,[23] which may be related to the fact that acids tend to produce at first a decrease in permeability (Osterhout, 1914); and (2) the fact that after a certain period the time of response begins to lengthen very rapidly. Now, the speed with which this falling off occurs varies with the different acids, and was found (at 0.02 N to 0.01 N) to follow the order:

Salicylic, oxalic > HCl, HNO₃, H₂SO₄ >
formic > lactic > malic > acetic.

[22] Oxalic acid was found to be so toxic that its curve cannot be considered with the rest.

[23] Frequently preceded by a heightened irritability.

The agreement of this order with that of cell penetration at these concentrations (Table VII) is striking, and indicates that as the ceptor is entered by acids, its irritability is immediately decreased.[24]

From this point of view it is comprehensible that in secondary sense cells, as of the vertebrate taste bud, we find nerve terminals in connection with the surface of the ceptor, not penetrating its interior.

We must regard this ceptor surface as a complex structure, and thus far the indication is that it contains both fatty substances and proteins.[25]
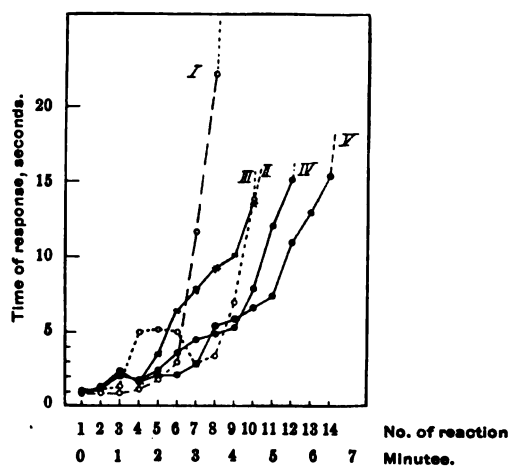
Fig. 6. Effect of successive stimulation on the time of response to 0.133 N salicylic acid; individual worms I to V.

SUMMARY.

1. The method employed for the study of acid penetration depended upon the selection of pieces of tissue from the edge of the mantle of the nudibranch *Chromodoris zebra* Heilprin; these pieces give reasonably uniform and reproducible results when the following conditions are observed: (a) the animals selected are of the same size and of the same kind and degree of coloration; (b) the

[24] The responses of the earthworm depend upon many scattered chemoreceptors, which are naturally not all influenced at exactly the same time.

[25] Compare Loeb (1912b), Osterhout (1913), and Loeb and Beutner (1913).

pieces of tissue are taken from corresponding locations, are treated in the same way, and submitted to the action of acids at a constant temperature. This tissue contains an intracellular blue substance, which is turned pink at about $p_H = 5.6$. The time required for this change when immersed in acid solutions is measured under conditions favorable to judgment of the precise moment of color change.

2. The relative penetrating speed of eighteen acids, measured over a range of dilutions, shows that two sets of factors are concerned in penetration. One of these is ionization, the other varies with the constitution of the acid. It is indicated that the cell surface is complex, and contains fatty substances, proteins, and probably other materials.

3. Comparison of these data with observations on artificial parthenogenesis (Loeb) suggests that the stimulus to membrane formation in the sea urchin egg depends upon the union of the acid with the surface of the egg, which is of quite different proportional composition from the cytoplasm of tissue cells.

4. Measurements of the stimulating power of various acids show, in the light of the penetration series, that the surface of chemoreceptors is of complex constitution, probably containing proteins and fatty substances; they show also that the process of stimulation involves the union of the stimulating agent with some constituent of the ceptor surface.

AGAR'S ISLAND, BERMUDA.

The following note was added while this paper was in press. In Harvey's later paper (*Z. physik.-chem. Biol.*, 1914, i, 463–478), which was until a few days ago unknown to me, it is shown that the dilution-*versus*-penetration curves for butyric and hydrochloric acids cut across each other very much as found for strong and weak acids with the tissue of *Chromodoris*. This agreement is rather striking and adds materially to the strength of the interpretation put upon these facts. With the testis epithelium of *Stichopus*, as with the integument of *Chromodoris*, the strong acid penetrates more easily from dilute solutions than does the fatty acid. The same condition appears in Harvey's experiments upon luminous bacteria (*Biol. Bull.*, 1915, xxix, 309). I must therefore disagree with his view that "living cells behave as if they were droplets of oil or fat solvents" (Harvey, 1914, *Z. physik.-chem. Biol.*, i, 476).

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THE RATE OF OXIDATIONS IN REVERSED ARTIFICIAL PARTHENOGENESIS.

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In 1913 Loeb[1] showed that by suitable treatment unfertilized eggs of the sea urchin *Arbacia*, in which development had been artificially initiated, could be caused to revert to their original inactive condition. This was accomplished by placing the eggs in solutions of sodium cyanide or chloral hydrate immediately after a preliminary treatment with butyric acid or an alkali or with alkaline hypertonic solution.

If the treatment with butyric acid or alkali only was applied, or if, after this treatment, the eggs were placed in the sodium cyanide or chloral hydrate solutions for an insufficient length of time, they would disintegrate on transference to normal sea water. On the other hand, eggs which had received a sufficiently long treatment with sodium cyanide or chloral hydrate, on transference to normal sea water, remained intact and could be either fertilized or, in some cases, induced to develop again by the usual methods of artificial parthenogenesis. Clearly, a reversal of the effects induced by the alkali or acid treatment had taken place.

The chloral hydrate and sodium cyanide treatments, as Loeb pointed out, cause the reversal of the induction of development by suppressing the developmental changes due to the alkali or acid treatment.

Until more is known as to the nature of the changes taking place in the egg on membrane formation, it is difficult to form any idea as to the nature of the reaction whose rate is decreased, but in view of the marked changes in the rate of oxidations in the egg coincident with fertilization or artificial membrane production, it seemed of interest to follow the course of the oxidations during the process of reversion described by Loeb.

[1] Loeb, J., *Arch. Entwcklngsmechn. Organ.*, 1914, xxxviii, 277.

In the following experiments the rate of oxidations was measured at different stages in the method used by Loeb,[2] and the results show clearly that after the reversal which he described the oxidations indeed continue at the normal rate existing in the unfertilized, untreated egg.

I. Method.

The method used in the determination of the rate of oxygen consumption was that of Siebeck.[3] This method possesses the great advantage that only relatively small amounts of egg suspension are required for a determination, and also that the rate can be ascertained at any desired moment during a determination. The readings are made by means of a water manometer, and the size of the apparatus is so arranged that 1 mm. pressure on the water manometer corresponds almost exactly to an oxygen consumption of 1 c. mm.

Heilbrunn[4] has recently objected that results by this method are not reliable, in that substances diffusing out of the egg into the solution cause a lowering of its solubility for oxygen, thus yielding a low result. Apart from the fact that an absolute value for the consumption of oxygen is neither necessary nor desired in such experiments, this source of error, though it undoubtedly exists, is shown to be negligible by means of simple controls suggested by the author of the method and readily carried out with *Arbacia* eggs.

In order to give some idea of the method of manipulation, a typical experiment will be briefly described.

A suspension of *Arbacia* eggs, after thorough mixing, was divided into six equal parts. Two of these parts were used for the control determination of oxygen consumption by the unfertilized eggs. The controls having been set aside, the remaining four portions were mixed, treated for $2\frac{1}{2}$ minutes with butyric acid (2.0 cc. 0.1 N acid to 50 cc. sea water), and immediately transferred to 50 cc. sea water + 0.4 cc. 0.1 per cent sodium cyanide

[2] Loeb, *Science*, 1913, xxxviii, 749; *Arch. Entwcklngsmechn. Organ.*, 1914, xxxviii, 277.

[3] Siebeck, R., Abderhalden's *Handb. biochem. Arbeitsmethoden*, 1915, viii, 33.

[4] Heilbrunn, L. V., *Science*, 1915, xlii, 615.

solution. This suspension was then divided into two equal portions, each one-third of the original amount of eggs taken. The first of these was washed four times in the sea water + sodium cyanide solution and finally divided into two equal portions, each one-sixth of the original amount, and placed in stoppered flasks. The other portion which had been treated with butyric acid was washed four times in normal sea water, and finally it too was divided into two equal portions. As a result of this manipulation, there were obtained six equal suspensions of eggs; two of these, the controls, had received no treatment, two had been treated with butyric acid and subsequently kept in the sea water cyanide mixture, while two had been treated with butyric acid and were subsequently kept in normal sea water in which they disintegrated in the course of a few hours.

Each portion received, as nearly as possible, an equal amount of washing, and, in order to equalize any error due to loss of eggs in the process, even the untreated eggs were subjected to the same washing, so that the rates of oxidation in the six final samples are fairly closely comparable. In order to reduce the volume of the suspension to the amount required for the oxygen determination, viz., 2.5 cc., an attempt was made at first to hasten the process by concentration in a slow speed centrifuge. It was found, however, that even when the greatest care was exercised, the eggs sometimes suffered through this treatment and the expedient was resorted to of allowing the eggs to settle by gravity in a centrifuge tube and decanting the supernatant sea water until the desired volume was obtained. The suspension was then transferred, by means of a wide-nosed pipette, to the flask in which the determination was carried out.

The determinations of oxygen consumption were in all cases carried out at a temperature of 25°C., kept constant to within 0.02°C. No matter what previous treatment the eggs had received, the final measurements were made in sea water in every case.

In all the experiments described in this paper, a similar method of manipulation was used.

II. Oxidations in Reversal.

The rates of oxidation in eggs treated with butyric acid and kept subsequently in sodium cyanide sea water are given in Table I. On removal from the sodium cyanide solutions, the eggs were, of course, washed and allowed to remain in normal sea water for about 1 hour before proceeding with the oxygen determination.

TABLE I

No. of experiment	1	2	3	4	5	6	7	8	9	10	
	Oxygen consumed, c. mm. per hr.										
Untreated eggs	27	25	30	30	18	28	20	24	10	17	17
Eggs treated with butyric acid	75	71	62	53	50	49	27	80	17	56	56
Eggs treated with butyric acid after about 19 hours in sea water + NaCN	53	40	52	29	21	37	21	22	10	17	17

Table I shows that, with the exception of the first three experiments in which the technique had not been so well developed and also in Experiment 6, the rate of oxidations after about 19 hours in the sodium cyanide solution, is nearly the same as for the untreated eggs.

The subsequent behavior of the eggs was followed for at least 1 day further. It was noted (1) whether the eggs remained normal on removal from the sodium cyanide solution, which was nearly always the case; (2) whether they could be fertilized, this being nearly always possible, a considerable proportion developing to swimming larvæ; and (3) whether they could be made to develop by repeating the treatment with butyric acid followed by hypertonic sea water, as in the ordinary method of artificial parthenogenesis. In some experiments a large percentage could be caused to develop to swimming larvæ by such treatment indicating a fairly complete reversal of the effects of the first butyric acid treatment, though in other experiments the reversal tested by this means proved less satisfactory. In such cases, however, this result found its explanation in the figure obtained for oxygen consumption, the reversion of the latter not being complete.· The completeness of the reversal was also tested by simple treatment

of the eggs for about 20 minutes with hypertonic sea water or removal from the sodium cyanide solution. If the eggs could be caused to develop by such treatment it was indicated that the effect of the original butyric acid treatment still persisted and that therefore the reversal was not complete. In two instances a large percentage of the eggs—about 15 per cent—underwent segmentation and developed to swimming larvæ on treatment in this manner. In the remaining experiments none or very few eggs responded to such treatment.

In some of the experiments the eggs which had remained about 19 hours in the sea water and sodium cyanide mixture were subjected again to the butyric acid treatment and their rate of oxidations was determined. Table II gives the result of these determinations.

TABLE II.

No. of experiment	6	7	8	9	10
	Oxygen consumed, c. mm. per hr.				
Untreated eggs	28	20	24	10	17
Eggs treated with butyric acid	49	27	80	17	56
Eggs treated with butyric acid after 19 hours in sea water + NaCN	37	21	22	10	17
Eggs treated with butyric acid after 19 hours in sea water + NaCN, but treated again with butyric acid	31	49	31	19	29

In all cases save in Experiment 6, the rate of oxidations rose again on retreatment with butyric acid, though the original rate was reached only in two instances. These results are further confirmation of the reversal of the effect induced by the first butyric acid treatment.

The rate of oxidations in eggs treated with ammonium hydroxide and kept subsequently in sodium cyanide sea water was also determined. These results are given in Table III. The method of manipulation was similar to that already described for the butyric acid treatment. The eggs remained for 20 minutes in a mixture consisting of 0.3 cc. 0.1 N ammonium hydroxide with 50 cc. sea water, being then transferred directly to the sodium cyanide solution.

TABLE III.

No. of experiment	11	12	·13	14	15	16
	Oxygen consumed, c. mm. per hr.					
Untreated eggs	19	18	24	11	16	9
Eggs treated with NH₄OH	26	28	37	49	48	31
Eggs treated with NH₄OH after about 19 hours in sea water + NaCN	28	38	35	15	17	11
Eggs treated with NH₄OH after about 19 hours in sea water + NaCN and treated again with NH₄OH	53	47	61	29	42	21

In the last three experiments the technique employed was better than in the three earlier ones. These results are substantially the same as those obtained in the experiments with the butyric acid treatment. The rate of oxidations, on removal from the cyanide solution, was approximately the original rate and increased again on retreatment with ammonium hydroxide.

After treatment with the alkali and transference to sea water the eggs disintegrated, though after about 19 hours in the sodium cyanide solution, on transference to normal sea water, they remained intact and normal. Such eggs on removal from the cyanide could be fertilized by sperm, and also in most cases caused to develop on retreatment with ammonium hydroxide or butyric acid and hypertonic sea water. When these eggs were placed directly into hypertonic sea water for about 20 minutes, after removal from the sodium cyanide solution, a small percentage segmented and a few even developed to the blastula stage, indicating that some effects of the original alkali treatment still remained and that reversal was not quite complete.

III. Fate of Eggs Which Did Not Receive the Cyanide Treatment.

The rates of oxidations in eggs which had received the acid or alkali treatment and had not been transferred to the cyanide solution but kept instead, until next day, in normal sea water, were also determined and the results are shown in Table IV.

TABLE IV.

No. of experiment	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Oxygen consumed, c. mm. per hr.													
Untreated eggs	30	30	18	28	20	24	10	17	19	18	24	11	16	9
Treated eggs (acid or alkali treatment) about 1–2 hours after treatment	62	53	50	49	27	80	17	56	26	28	37	49	48	31
Treated eggs next day	39	58	62	31	34	38	20	15	52	80	93	23	53	19

It will be noted that the figures for the oxidations on the second day are somewhat inconsistent. In some experiments the rate of oxidations has fallen to a figure not much greater than the original rate, while in other experiments it remains as high as or even higher than the increased rate due to membrane formation by acid or alkali treatment.

There is also considerable variation exhibited in the magnitude of the response to the acid or alkali treatment, in some experiments an increase in oxidations of about 400 per cent is noted, while in some the increase is less than 100 per cent.

The explanation for some of these apparent inconsistencies is fairly simple and lies in the fact that the magnitude of the response of sea urchin eggs to a given treatment by acid or alkali is not always the same, the variations being due to differences in sensitivity in the eggs of individual females and perhaps also to differences in room temperature during the several experiments. This accounts for the varying percentage increase in the rate of oxidations on a given treatment with acid or alkali and also for an unequal resistance to the disintegrating effects of such treatment, observed in the eggs of different females. It also accounts for differences observed in the rate of oxidations in untreated eggs preserved for some time in sea water in the laboratory. These frequently show a considerable spontaneous increase in a relatively short time, as will be seen from the figures given in Table·V.

TABLE V.

No. of experiment	8	9	10	11	12	13	14	15	16
	Oxygen consumed, c. mm. per hr.								
Untreated eggs	24	10	17	19	18	24	11	16	ᶜ
Untreated eggs kept over night in normal sea water	29	17	17	67	36	80	23	26	1ᶜ

In all these experiments except No. 10 a considerable increase has taken place. The magnitude of this increase, however, shows considerable variation.

IV. The Spontaneous Increase in Oxidations in Unfertilized Eggs

In order to account more fully for the apparent inconsistency of the results in Tables IV and V, it is necessary to consider in some detail the spontaneous increase in oxidations in the eggs on standing. An endeavor was made to ascertain what factor is responsible for the phenomenon.

Loeb[5] showed that some of the eggs of certain females of the sea urchin *Strongylocentrotus purpuratus* formed fine gelatinous membranes spontaneously, when allowed to remain for some time about 24 to 48 hours, in sea water at relatively low temperature He found that these eggs sometimes segment, and if kept at a low temperature may even reach the sixteen cell stage. Loeb also found that if eggs which form membranes spontaneously are treated with hypertonic sea water, as in the usual method of artificial parthenogenesis, some will develop to larvæ.

In the eggs of *Arbacia* such spontaneous membrane formation is hardly ever observed. This may be due to the fact that in *Arbacia* eggs the membrane lies much more closely on the surface of the egg than is the case in *Strongylocentrotus*, and is therefore more difficult to observe. It is certainly true of the fertilization membrane and even more true of the membrane formed after the butyric acid treatment. A spontaneous segmentation is sometimes noted in *Arbacia* eggs, but this never goes beyond the two or three cell stage. It is possible, therefore, that spontaneous membrane formation or some spontaneous alteration of

[5] Loeb, *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvi, 626.

the cortical layer also occurs, in the eggs of some females, in *Arbacia*.

Artificial membrane formation in *Strongylocentrotus* is always accompanied by a considerable increase in oxidations, whether the membranes have been called forth by butyric acid or by cytolytic agents such as saponin.[6] While the point has not been tested, it is reasonable to suppose that spontaneous membrane formation is also accompanied by a considerable increase in oxidations.

Loeb suggested that the spontaneous membrane formation in *Strongylocentrotus purpuratus* might be caused by the alkalinity of the sea water and it was thought desirable to ascertain whether the spontaneous increase in oxidations in *Arbacia* eggs takes place more readily in a slightly alkaline than in a slightly acid sea water. To this end the following simple experiment was carried out. Eggs in equal quantities were placed in a number of finger bowls. One-half of these bowls contained an artificial sea water made by mixing 100 cc. 0.5 M NaCl, 2.2 cc. 0.5 M KCl, 1.5 cc. 3/8 M CaCl₂, 7.5 cc. 3/8 M MgCl₂, and 3.5 cc. 3/8 M MgSO₄. This solution is acid to neutral red, while the normal sea water is slightly alkaline. All the various lots of eggs received an equal amount of washing. At intervals the rates of oxidations in eggs taken from the normal and from the artificial sea water were compared, the determination in both cases being performed in normal sea water. For this purpose the eggs from the artificial sea water were washed in normal sea water immediately before making the determination. The results are contained in Table VI.

[6] Loeb, J., and Wasteneys, H., *J. Biol. Chem.*, 1913, xiv, 469.

TABLE VI.

Length of time eggs had remained in solution.	Normal sea water (slightly alkaline).		Artificial sea water (slightly acid).	
	Oxygen consumed per hr.	Condition of eggs.	Oxygen consumed per hr.	Condition of eggs.
hrs.	c. mm.		c. mm.	
3	12	Normal.	10	Normal.
20	19	Majority normal, some disintegrated.	10	"
26	19	Majority normal, some disintegrated.	13	"
44	57	Agglutinated and disintegrated.	27	Majority norma -l, some cytolyzed.
1½	11	Normal.	9	Normal.
19	11	"	10	"
44	73	All disintegrating.	19	Majority norma -l, some disintegra -at- ing.
1	10		9	
5½	19		18	
8½	17		16	
24	34	Many disintegrating.	37	.
47	49	All "	29	Majority normal.
19	8		9	
29	31	Some disintegrating.	16	Normal.

These results, while incomplete, indicate that alkalinity pla_____ys an important rôle in the spontaneous increase in oxidations in t___ he sea urchin egg, and though more experiments are required to determine its degree of responsibility, it is evident that the spo_____n-taneous disintegration of the eggs in sea water can be stron____ly inhibited by reducing the alkalinity.

The rôle played by the alkalinity of the sea water is also str___ k-ingly illustrated in experiments with butyric acid treatment of the eggs. In these experiments the butyric acid solution was made with artificial sea water possessing a faintly acid react___n instead of ordinary sea water which is faintly alkaline. The e____gs were transferred from the butyric acid solution directly to a__ ti-

ficial sea water or to normal sea water as the case might be, and the relative increase in the rate of oxidations was determined 1 hour later. The results are given in Table VII.

TABLE VII.

Treatment.	2½ min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water.	5 min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water.	5 min. in 1 cc. 0.1 N butyric acid to 50 cc. artificial sea water.	3 min. in 2 cc. 0.1 N butyric acid to 50 cc. artificial sea water.
Untreated eggs........	21.5	11	7	11
Treated eggs transferred to normal sea water..............	26.0	18	16	46
Treated eggs transferred to artificial sea water..........	16.0	9	6	11

When the eggs are transferred from the butyric acid solution to the faintly acid artificial sea water, the membranes are not formed so well as when the eggs are transferred to the slightly alkaline sea water. There is also some loss in the treatment with butyric acid in artificial sea water as the eggs in such a solution have a strong tendency to stick to the sides of the vessel. This accounts for the decrease in the rate of oxidations shown by the treated eggs when transferred to artificial sea water.

In the slightly acid sea water no increase in the rate of oxidations takes place following butyric acid treatment. The increase on transference to normal sea water shows, however, that the butyric acid treatment was sufficient for the purpose. The presence of excess of hydroxyl ions seems, therefore, to be necessary for the activation of the processes resulting in increased oxidations.

This result was forecasted by Loeb[7] who found that eggs of *Strongylocentrotus purpuratus* when treated with butyric acid dissolved in neutral 0.5 M Ringer solution and transferred to neutral 0.5 M Ringer *per se*, formed only abnormal membranes and neither disintegrated nor developed. He suggested that the butyric acid treatment had not brought about the usual increase in oxidations but was inclined to attribute the abnormal behavior to causes other than hydroxyl ion concentration.

[7] Loeb, *Biol. Bull.*, 1915, xxix, 103.

One other possibility suggested itself in connection with the spontaneous increase in oxidations. It was observed that, when a considerable quantity of eggs remains in contact with only a small amount of sea water, a few of the eggs occasionally form membranes. It was also noticed that under such conditions the eggs appeared to disintegrate earlier than eggs which are less closely packed. This might be due to the presence, in considerable amount, in the surrounding sea water, of substances exuded by the eggs, which induced that critical change in the cortical layer of the egg responsible for membrane formation. Such eggs should exhibit an increased rate of oxidation.

In order to test this point, a suspension of eggs was equally divided. One portion was placed in a small amount of sea water under conditions of minimal evaporation while the other equal portion was placed in a relatively much larger quantity of sea water which was frequently renewed. Determinations of the rate of oxidations were then made at intervals, with the result given in Table VIII.

TABLE VIII.

No of experiment..............	1			2			
No. of hours under given condition.	4	23	26	4½	9	22	27½
	Oxygen consumed, c. mm. per hr.						
Eggs kept in a small quantity of sea water.........	23	73	71	9	10	21	32
Eggs kept in a large quantity of sea water which was frequently renewed.	22	38	52	10	24	25	48

It is evident from these figures that the spontaneous increase in the rate of oxidations continues in spite of dilution and removal of the egg exudates and is not even markedly inhibited thereby.

As a further control on the behavior of eggs on standing in sea water, a few determinations were made of the rate of oxidations in normal untreated eggs which had been kept over night in the sea water and sodium cyanide mixture. It is hardly necessary to state that the eggs were well washed in sea water and allowed to stand for some time after removal from the cyanide solution

before proceeding with the determination. These results are given in Table IX.

TABLE IX.

No. of experiment	14	15	16
	Oxygen consumed, c. mm. per hr.		
Untreated eggs	11	16	9
Untreated eggs on following day	23	26	16
Untreated eggs kept over night in NaCN solution	12	19	11

The changes responsible for the spontaneous increase in oxidations in unfertilized eggs on standing are evidently inhibited by preserving them in the sodium cyanide solution. This agrees with the old observation of Loeb and Lewis[8] that in potassium cyanide the life of unfertilized sea urchin eggs is prolonged.

On subjecting these eggs which had remained over night in the cyanide solution to treatment for 20 minutes with 0.3 cc. 0.1 N ammonium hydroxide in 50 cc. sea water, the oxidations were increased to an amount approximating that reached by eggs from the same lot when treated on the previous day, thus proving that the cyanide treatment had kept the eggs intact. This is shown by the results given in Table X.

TABLE X.

No. of experiment	14	15	16
	Oxygen consumed, c. mm. per hr.		
Untreated eggs	11	16	9
Eggs after NH₄OH treatment	49	48	31
Untreated eggs kept over night in NaCN solution	12	19	11
Eggs kept over night in NaCN solution and then treated with NH₄OH	34	42	39

In some of the experiments described, it was thought desirable to follow further the fate of eggs which had received the butyric

[8] Loeb, J., and Lewis, W. H., *Am. J. Physiol.*, 1901-02, vi, 305.

acid treatment. Accordingly, the treated eggs were preserved until the following day and the rate of oxidations was once more determined. A comparison of the figures obtained for treated eggs with those for untreated eggs kept during a similar period is of interest, especially in connection with the question of spontaneous oxidations. These results are given in Table XI.

TABLE XI.

No. of experiment.	Time after treatment.	Untreated.	Treated.	Condition of treated eggs on second day.
		Oxygen consumed per hr.		
		c. mm.	c. mm.	
8	1 hr...............	24	80	
	Next day........	29	38	All disintegrated.
9	1 hr...............	10	17	
	Next day........	17	20	" "
10	1 hr...............	17	56	
	Next day........	17	15	" "
11	1 hr...............	19	26	
	Next day........	67	52	Some intact.
12	1 hr...............	18	28	
	Next day........	36	80	Majority intact.
13	1 hr...............	24	37	
	Next day........	80	93	70 per cent intact.
14	1 hr...............	11	49	
	Next day........	23	23	Majority disintegrated.
15	1 hr...............	16	48	
	Next day........	26	53	" "
16	1 hr.	9	31	
	Next day........	16	19	" "

The untreated eggs were nearly always intact on the second day. It will be noticed that in some experiments the treated eggs show a diminution in oxidations on further standing, while in other experiments an increase occurs.

These results, at first glance somewhat anomalous, may be explained as follows: If the idea, that the spontaneous increase in oxidations is due to the changes in the cortical layer which underlies membrane formation, is correct, then it is also probable that such alterations in the cortical layer of the egg will continue, influenced by the excess of OH ions in the sea water, even after membrane formation has been induced by acid treatment. In such a case the rate of oxidations will also continue to increase. Thus, in Experiments 11 and 13, the oxidations were not greatly raised by the butyric acid treatment. It happened, however, that in the untreated eggs the oxidations increased spontaneously to a high figure; consequently, in the treated eggs, the oxidations continued to increase, also spontaneously, until a correspondingly high figure was attained. In the other experiments, with the exception of No. 12, the oxidations increased considerably on treatment. Such eggs show no or only very slight further increase in oxidations on the following day, the maximum having already been reached as a result of the acid treatment. Generally, owing probably to disintegration, the oxidations have again diminished on the next day, by an amount roughly proportional to the degree of disintegration.

Another explanation is also possible though perhaps less probable, and is as follows: Warburg and Meyerhof[9] found that the oxidations in unfertilized sea urchin eggs are only slightly less after disintegration than before. Warburg[10] found that when fertilized eggs are caused to disintegrate, the rate of oxidations returns to the value for an equal quantity of unfertilized eggs. He concluded that the increase in oxidations on fertilization is bound up in the existence of the fertilization membrane. This idea resembles somewhat the *Hauptathmung* and *akzessorische Athmung* of Battelli and Stern.[11] Warburg[12] showed that the membrane called forth by the butyric acid treatment acts like the fertilization membrane in increasing the rate of oxidations. Assuming that on disintegration the rate of oxidations in the treated eggs similarly falls again to the rate in the unfertilized eggs, which

[9] Warburg, O., and Meyerhof, O., *Arch. ges. Physiol.*, 1912, cxlviii, 295.
[10] Warburg, O., *Arch. ges. Physiol.*, 1914, clviii, 189.
[11] Battelli, F., and Stern, L., *Biochem. Z.*, 1914, lxvii, 443.
[12] Warburg, *Z. physiol. Chem.*, 1910, lxvi, 305.

is probable, and assuming that the spontaneous increase in oxida
tions in unfertilized eggs is not caused by spontaneous membran
formation but by the action of the OH ions of the sea water inde
pendently of membrane formation, the results in Table XI ca
be readily explained.

In Experiments 12 and 13 the oxidations are slightly raised b
the acid treatment and the spontaneous increase in the untreate
eggs next day is considerable. A spontaneous increase has sim
larly occurred in the treated eggs, and as the eggs had not dis
integrated the figures remained high. In the remaining experi
ments in which the treated eggs had disintegrated on the secon
day, the increased rate of oxidations has diminished again to
value corresponding to that for the untreated eggs. Where n
spontaneous increase has taken place, the increased rate due t
treatment has dropped to the original value. In those cases wher
some spontaneous increase took place the final value on the secon
day for both treated and untreated eggs agrees closely.

V. What Causes the Reversal Effects?

It has been shown that after the reversal of the action of butyri
acid as demonstrated by Loeb, the rate of oxidations is the sam
as the rate existing before treatment, and the several question
subsidiary to the main issue have also been answered fairl
satisfactorily.

The next question for consideration would appear to be, Ho
does the sodium cyanide solution accomplish the reversion? W
know that it suppresses oxidations and development, but there
no a priori reason why a temporary suspension of induced ce
activities should result in a reversion to the reactions of an unfe
tilized egg. It is to be remembered, in this connection, that, ı
Loeb showed, chloral hydrate acts similarly to sodium cyanic
in causing a reversion, and the chloral hydrate compared to sodiu
cyanide has a relatively slight effect in depressing the oxidatioı
in sea urchin eggs. The following experiments carried out wit
Arbacia eggs serve to illustrate this.

TABLE XII.

	Relative oxygen consumption.
Fertilized eggs in normal sea water............................	1.00
" " " 50 cc. " " + 0.22 cc. 0.1 per cent NaCN.	0.15
" " " 50 " " " " 0.22 " 0.1 " " "	0.27
" " " 50 " " " " 0.15 " 0.1 " " "	0.20
" " " 40 " " " " 10.00 " 0.5 " " chloral hydrate..	0.67
Fertilized eggs in 42.5 cc. sea water + 7.5 cc. 0.5 per cent chloral hydrate...	0.61
Fertilized eggs in 44.0 cc. sea water + 6.0 cc. 0.5 per cent chloral hydrate...	0.73

These experiments were made in the course of another investigation and the concentrations are not those most suitable for the reversion of the effects induced by butyric acid. The concentrations used in the reversal experiment are 0.4 cc. 0.1 per cent sodium cyanide or 5 cc. 0.5 per cent chloral hydrate per 50 cc. sea water mixture, consequently the depression of oxidations during the reversal process is probably less for chloral hydrate and greater for sodium cyanide than is shown by the above figures.

It is probable, therefore, that the main factor concerned in bringing about reversion is the suppression of the developmental processes, the lowering of the rate of oxidations being merely the means of suppressing cell division.

In conclusion, the writer wishes to express his thanks to Professor Jacques Loeb for advice and criticism.

SUMMARY.

1. The rate of oxidations was determined in sea urchin eggs in which development had been initiated and later reversed by the methods of Loeb.

2. It was found that the rate of oxidations, which increases after initiation of development, returns, after reversal, to approximately the original rate of the untreated eggs.

3. It was found that on retreating the reversed eggs so as to initiate development once more the oxidations are again increased.

4. Eggs in which initiated development had been reversed could be fertilized by sperm, and also in most cases could be caused to develop by the usual methods of artificial parthenogenesis.

5. The oxidations in eggs in which development had been initiated but not reversed were studied, as were also the oxidations in untreated eggs from the same batch.

6. It was observed that the rate of oxidations in untreated eggs rose spontaneously on standing, sometimes showing a 300 per cent increase.

7. The probable cause of the spontaneous increase was found in the alkalinity of the sea water.

8. It was shown that the oxidations do not increase in eggs treated with butyric acid when transferred to faintly acid sea water.

9. The cause of the reversal is discussed, and it is shown to lie mainly in the suppression of developmental processes.

UNITS OF REFERENCE FOR BASAL METABOLISM AND THEIR INTERRELATIONS.

By C. R. MOULTON.

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The data upon which this paper is based were collected in connection with the general Use of Food Experiment which has been conducted for the past 8 years at the Agricultural Experiment Station of the University of Missouri. The animals studied were of the Hereford-shorthorn cross of beef cattle. In addition to extensive feeding and slaughtering trials and chemical analyses, maintenance and digestion trials were run and measurements were made of the surface area, blood weight, body weight, empty body weight, fat-free empty weight; and total protoplasmic tissue. The total nitrogen by analysis was used as a measure of the total protoplasmic tissue.

The data have already been given[1] in some detail and it has been pointed out that the condition of the cattle influences the relations between body weight and empty weight, and between empty weight and weight of nitrogen, surface area, and blood weight and volume. The relations are, however, constant for like conditions. It is the purpose here to discuss part of the data there presented together with a few additional data. It is hoped that some light will be thrown upon the general question of what is the best unit of reference for basal metabolism and what are the relations, if any, between these units. The latter part of the question will be considered first.

[1] Trowbridge, P. F., Moulton, C. R., and Haigh, L. D., The Maintenance Requirement of Cattle, *Missouri Agricultural Experiment Station, Research Bulletin 18*, 1915.

I.

Perhaps the simplest conception of the needs of animals for foo**ᴏᴏᴏ**
is based upon the generally observed fact that larger animal **Iᴀᴄʟᴀ**
need more food than smaller animals and that the needs are **ɪᴀ** a
roughly proportional to the mass, being about the same for eacᴎ **ᴏᴄ**
unit of mass for like animals under like conditions. The activ**ɪᴄɪᴠ**
part of this unit of mass may be a large or a small part of the uni **ᴏᴄᴜɴɪ**
in the case of beef cattle, as the author has shown elsewhere. **ᴏᴄᴇ**
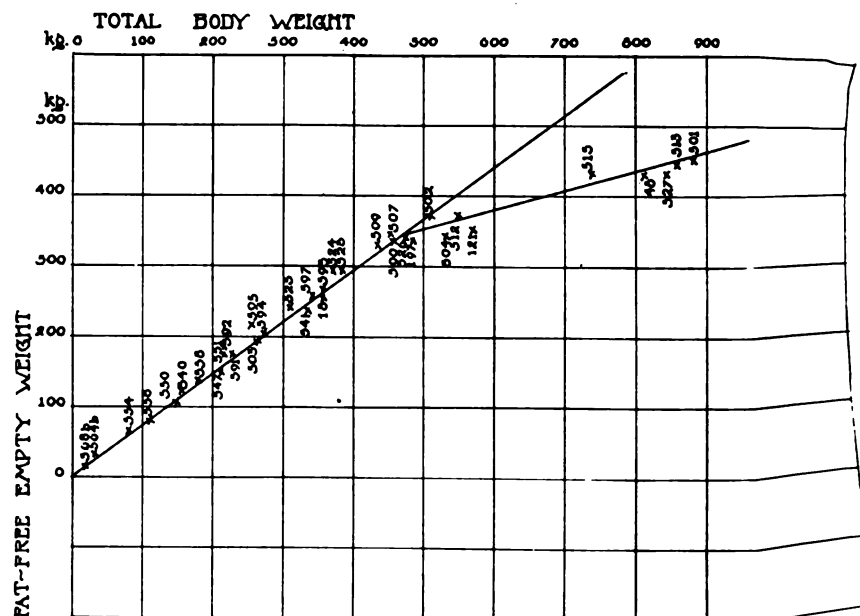
Fɪɢ. 1. Relation between fat-free empty weight and total body weig——ᵗ
of 35 beef cattle.

The amount of the nitrogenous, or protoplasmic, tissue is   a
measure of the active machine within the animal. The percen t-
age of this tissue may be 50 per cent more in one beef steer tha——
in another, depending upon the relative fatness of the animals.

Fig. 1 shows the relation between total body weight and fa——t-
free empty weight of 35 beef steers. The animals range in a——
from calves at birth to 4 and 5 year olds, and in weight from 17— .7
to 883.5 kg. The condition, or fatness, varied from emaciatic——ɴ

to extreme fatness. In spite of these variations a fairly constant ratio is shown for the animals of poor to good condition. Only the prime and overdone steers show a greatly decreased proportion of fat-free weight. For the purpose of the general experiment most of the animals had been divided into three groups. Group I was full fed from birth, Group II was fed for maximum growth without the laying on of appreciable fat, and Group III was fed for retarded growth or to represent poor farm conditions.
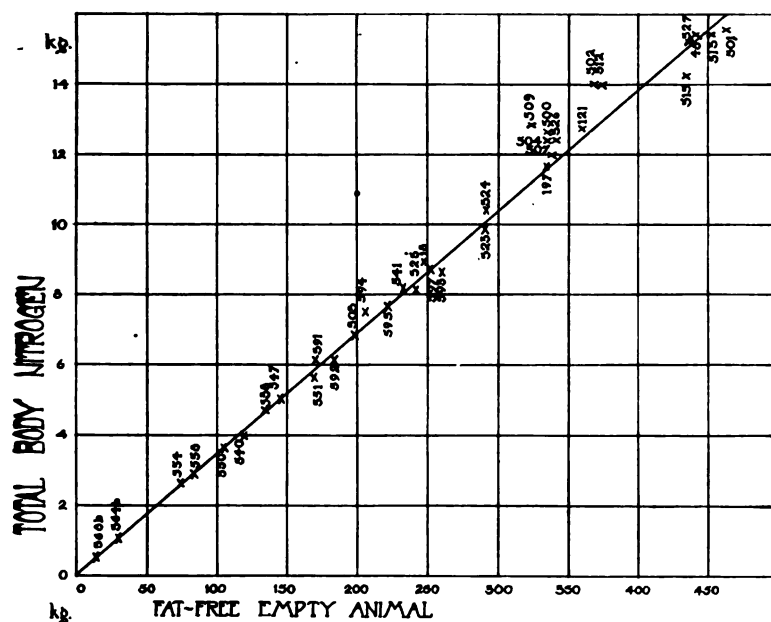
Fig. 2. Relation between total body nitrogen and fat-free empty animal.

The other animals were assigned to the groups defined according to their relative condition. The calves at birth are distinguished by a letter b after the number. It is the Group I animals 2 years old and over that show the decreasing proportion of fat-free weight and increasing proportion of fat. It is thus made evident that, while the unit of mass may be fairly uniform for the thin and medium fleshed animals and may represent about the same amount of active machine, this unit of mass has a different composition as the animal gets fatter.

In order to show that the fat-free empty weight is a good measure of the active machine there has been plotted in Fig. 2 the relation between the total nitrogen by analysis and the fat-free empty weight. This relation is very constant, 1 kg. of body nitrogen representing 29 kg. of fat-free animal irrespective of age, condition, or weight. In Fig. 3 the same relation is shown between the total nitrogen excluding that in the blood and the fat-free empty weight. 1 kg. of nitrogen exclusive of that in the blood
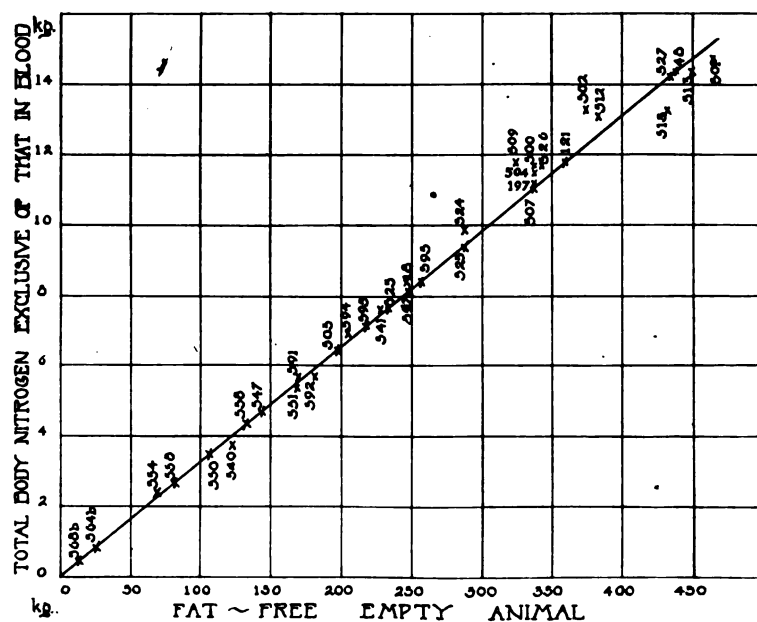
Fig. 3. Relation between total body nitrogen exclusive of that in the blood and fat-free empty weight.

represents 30.5 kg. of fat-free animal. The relation between the nitrogen excluding that in the blood, or hide, or bones, or any combination of the three, and the fat-free empty weight could be shown here. The relation is just as constant as those shown, one unit of nitrogen representing a larger unit of fat-free weight as more nitrogen is excluded from the total. The reason for excluding any nitrogen would be on account of its being inactive in the body metabolism. Since we have no standards of activity for the different divisions of the nitrogenous tissue the author has

considered the total nitrogen to be a good measure of the total active protoplasmic tissue.

Relation of Surface Area to Body Weight.

A better conception of the basal needs of animals for food can be obtained from a comparison of the relative surface areas of the animals. Since Rubner[2] and Richet[3] presented evidence to show that the heat production of living animals was propor-
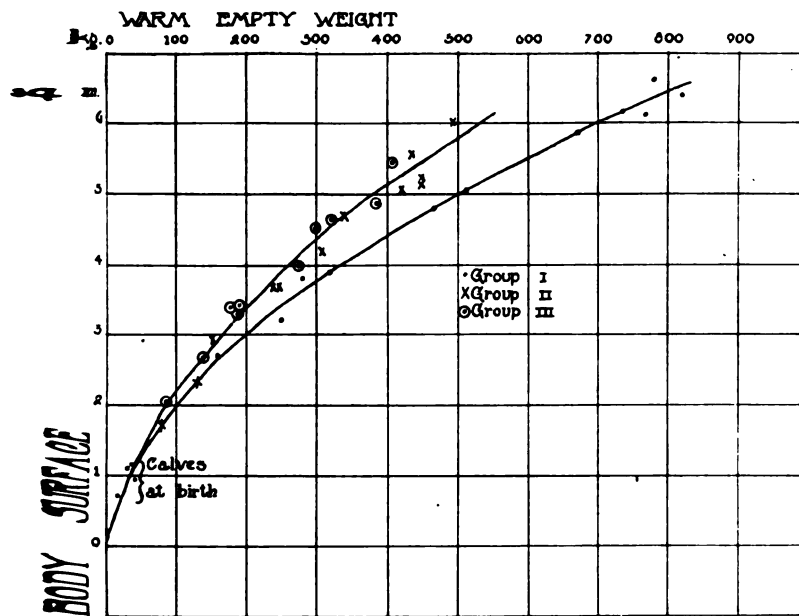
Fig. 4. Relation between body surface and warm empty weight.

tional to the body surface, this has been a much used unit of reference. The relation between body weight and surface area is derived from the mathematical law that the surfaces of like geometrical figures are proportional to the two-thirds power of the volumes. The specific gravity of like animals being about constant, it develops that $S = KW^{\frac{2}{3}}$, where S is the surface area

[2] Rubner, M., Z. Biol., 1883, xix, 545.
[3] Richet, C., Arch. de physiol., 1885, xvii, pt. ii, 284.

and W the body weight. The value of K has been worked out
for a number of animals from a few more or less limited obser-
vations. The author[1] has compared actually observed values
of S and W in 35 steers, and has found that K varies from
7.32 to 10.48 according to the fatness of the animal. For Group
III animals it averaged 9.92, and for Group II animals 9.37.
In Group I animals two classes were found; the young animals
showed an average value for K of about 8.57, while the older
animals showed an average value of 7.65. The relations found

Fig. 5. Relation between body surface and fat-free empty weight.

are shown in Fig. 4, where one curve represents the relation for the
animals in thin or fair condition, and a second curve the relatio.
for the fat animals. The empty weight is used in place of th
live weight in order to eliminate the influence of the amount c
food and food residues upon the weight. The four calves a
birth were considered to be representative of all groups at birth
The curves have the general appearance of power function curve
    The relation between surface area and fat-free weight is show
in Fig. 5. The straight line is drawn to show that the functio

is by no means a straight line function, as it is in the case of nitro-
gen and fat-free weight.   There are not shown here, however, two
different curves, the effect of the fat animals being simply to cause
a wider dispersion of values toward the right hand end of the curve.

In order to determine what relation exists between surface area
and body weight recourse can be had to logarithmic cross section
paper.   Fig. 6 shows the values found for surface area and empty
weight of 37 cattle.   If we disregard the values for the Group I
cattle, it is seen that the points arrange themselves well along a
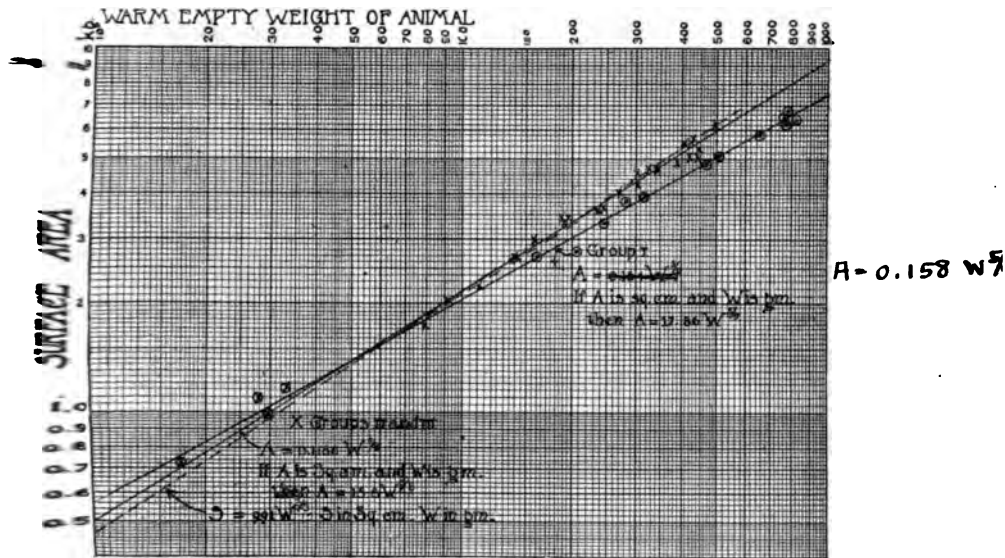
FIG. 6. Relation between surface area and warm empty weight plotted
on logarithmic cross section paper.

definite straight line.   Such straight lines on logarithmic paper
are an indication of a power function of the general formula
$Y = KX^n$.   The inclination or tangent gives the value of n.   If
the line is followed to the point where it crosses the ordinate aris-
ing from the abscissa $X = 1$, K can be read off directly, or it can
be calculated at the point where $X = 10$ or $X = 100$.   The for-
mula for the curve was thus found to be $Y = KX^{\frac{2}{3}}$.   If A is the
surface area in square meters and W the empty weight in kg.,
$A = 0.1186 W^{\frac{2}{3}}$.   The formula used at present is $S = KW^{\frac{2}{3}}$ The

exponent $\frac{2}{3}$ is $\frac{16}{24}$, and the exponent $\frac{5}{8}$ is $\frac{15}{24}$. This is a differei only $4\frac{1}{6}$ per cent. However, the line S = KW$^{\frac{1}{2}}$ does not fi facts shown. The dotted line, which was drawn at the inclin of $\frac{2}{3}$ through the greater part of the points, lies well below a calves at birth and above most of the old animals. It doe divide the points as uniformly as does the line A = KW$^{\frac{1}{2}}$.

For the fat cattle (Group I) the difference between the obs< formula and the one in present use is greater yet, for Fig. 6 ε the formula A = 0.134 W$^{\frac{1}{2}}$. This shows a smaller surface

$$A = 0.158 \, W \, \frac{2}{3}$$
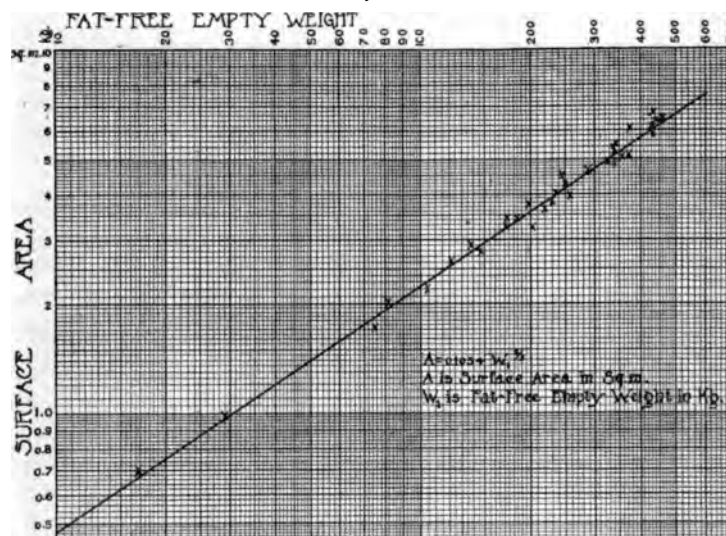
FIG. 7. Relation between surface area and fat-free empty weight p on logarithmic cross section paper.

relative to the weight for very fat cattle and is precisely the ε tion expected. Fatter animals have a rounder conformity thus a smaller surface area for a given volume. The for S = KW$^{\frac{1}{2}}$, then, does not fit the facts for very fat animals, ε large error will be introduced by using it for beef cattle.

Fig. 7 shows the data for surface area and fat-free ei weight treated in a similar manner. At a given weight the G I animals gravitate toward the bottom of the values sh while the Group II and Group III animals are toward the

But since the differences are slight and some errors are to be expected (see below), all the values are treated together. The points are distributed with considerable uniformity along a line at an inclination of $\frac{2}{3}$. The formula then in the same units is $A = 0.1034\,W_1^{\frac{2}{3}}$, where W_1 is the fat-free empty weight. On the basis of the fat-free animal the relation $S = KW^{\frac{2}{3}}$ does hold, and for beef animals containing a negligible amount of fat the usual formula can consequently be used without appreciable error.

Methods.

A critical discussion of the methods of obtaining the data plotted in Fig. 6 may be of service in showing the reliability of the conclusions. The cattle were weighed immediately before killing. They were then stunned, hoisted by the hind feet, and thoroughly bled into tared containers. The bleeding was assisted by pumping the fore legs. Blood dripping from the carcass later was caught and weighed. The cattle were then skinned. After disembowelling, the stomachs, intestines, and bladder were weighed full and then emptied, cleaned, and weighed again. The contents thus found for the stomachs, intestines, and bladder were subtracted from the live weight to get the warm empty weight. Errors of any moment could hardly creep in here. The entire hide, or sometimes the half hide from one-half of the carcass, which had been split by an expert, was traced on a large sheet of paper made by pasting heavy glazed wrapping paper together. The hide could be made to lie very flat and, with a little care, an accurate tracing could be made. Later these tracings were measured. The earlier measurements were made by drawing rectangles and triangles on the hide map. Later maps were cut into rectangles and triangles and fitted into a large rectangle the area of which could be easily determined. The latest measurements were made directly on large sheets of cross section paper upon which the hide outline had been traced. Of the errors due to the different methods used little can be proved except in the case of the last. Here two different cross section papers were used which caused the author to note discrepancies in the variation of K calculated from $S = KW^{\frac{2}{3}}$. These variations were expected to follow certain rules. The writer then checked up the

cross section paper and found that the second lot used was correct, but that the first lot gave an area too low by at least 5 per cent. The writer can thus predicate errors of like or even greater size due to some of the earlier methods used. Unfortunately these errors cannot be measured, as the meter sticks used were not kept or standardized. The writer feels that errors of as much as 5 per cent, or even 10 per cent, might have crept into the determinations of surface area. These could easily account for nearly all the points lying off the curves shown in Figs. 4, 5, 6, and 7.

FIG. 8. Relation between body surface and total body nitrogen.

The Relation of Surface Area to Body Nitrogen.

It has just been shown that there is a relation between the fat-free empty weight and surface area of cattle, and it was shown earlier that the fat-free animal had a constant percentage of nitrogen. It should, therefore, be expected that a relation exists between the surface area and total nitrogen in the body. The values found are plotted in Fig. 8. The points are arranged uniformly along the curve shown. Fig. 9 shows the same relation
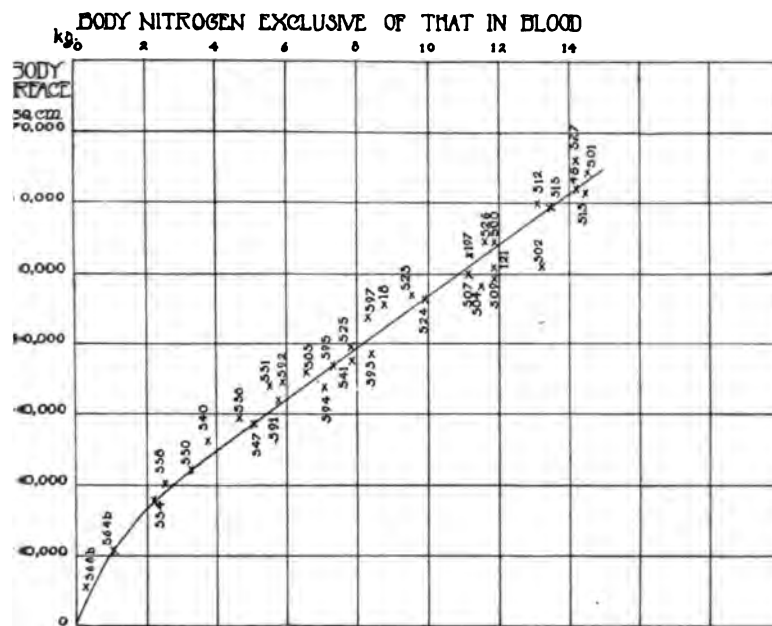
FIG. 9. Relation between body surface and total body nitrogen exclu-
ive of that in the blood.
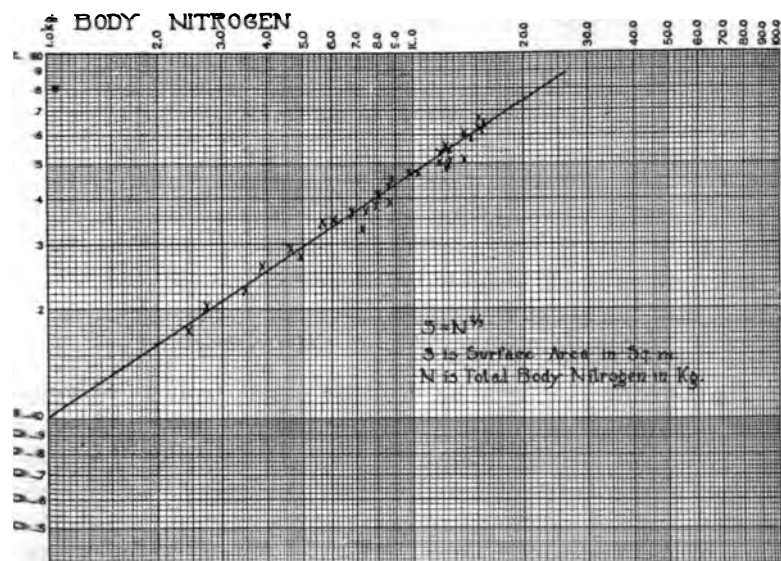
FIG. 10. Relation between body surface and total body nitrogen plotted
on logarithmic cross section paper.

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between body surface and body nitrogen exclusive of that in the
blood. Practically no change is made in the relative position of
the points. The values of Fig. 8 are shown plotted on logarithmic
paper in Fig. 10. The inclination of the line is $\frac{2}{3}$ and the value of
K is 1. So the formula found is $S = N^1$ where S is the surface
area in square meters and N is the total body nitrogen in kg.
This is a confirmation of the curve $A = KW_1^1$, as the relation of
N to W_1 is constant.

The Relation of Blood to Body Weight.

The tissues and organs are dependent upon the blood for food.
A larger animal has more blood than a smaller animal and has a
greater need for food. In order to see if a unit of body requires a
definite blood supply for its uses, a study will now be made of the
relations of blood supply to body weight. Fig. 11 shows the rela-
tion of blood weight to empty weight. The three groups of ani-
mals are distinguished by different marks. There is a marked
tendency for the points to arrange themselves along a line but with
rather large variations from an average. Four of the oldest and
fattest Group I animals line up along the dotted line far to the
right of the average curve. This shows that for the very fat ani-
mals the blood supply does not keep pace with the body weight.
This is to be expected since there is a large proportion of fat in
these animals and the blood supply for the fatty tissue is small.
Fig. 12 shows the relation of blood supply to fat-free empty
weight. The relation is more nearly constant, 1 kg. of blood
supplying 17.6 kg. of fat-free animal. However, the very fat
animals lie above the curve along the dotted line, showing a
greater proportion of blood to fat-free weight.

The Relation of Blood to Body Nitrogen.·

Fig. 13 shows the relation of blood supply to body nitrogen.
The three groups are distinguished by different marks. Most of
the values are arranged along a straight line showing that 1 kg.
of body nitrogen excluding that in the blood needs 1.6 kg. of blood
to feed it. The very fat Group I animals again show the pres-
ence of relatively more blood for each unit of body nitrogen
(protoplasmic tissue) to be supplied. Fat living seems to result
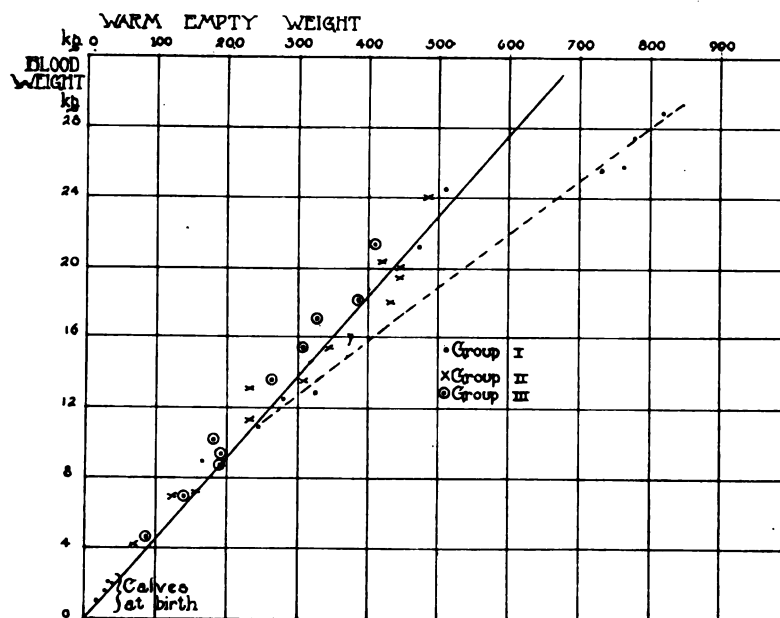
FIG. 11. Relation between blood weight and warm empty weight.
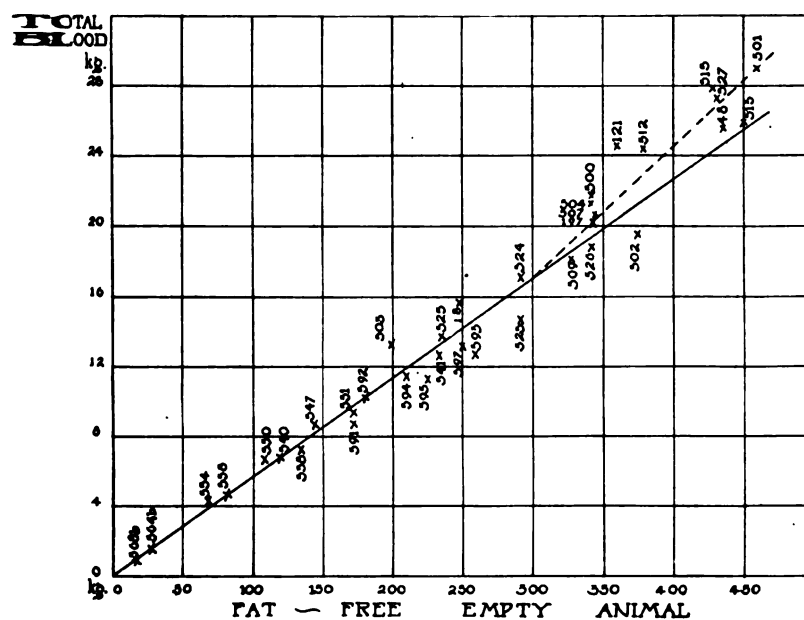
FIG. 12. Relation between blood weight and fat-free empty weight.

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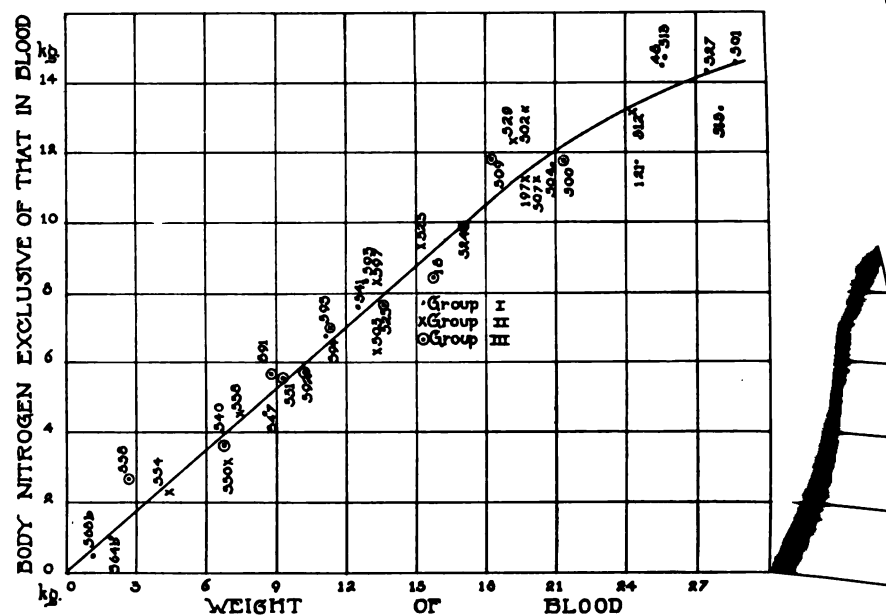
FIG. 13. Relation between total body nitrogen exclusive of that in the blood and blood weight.
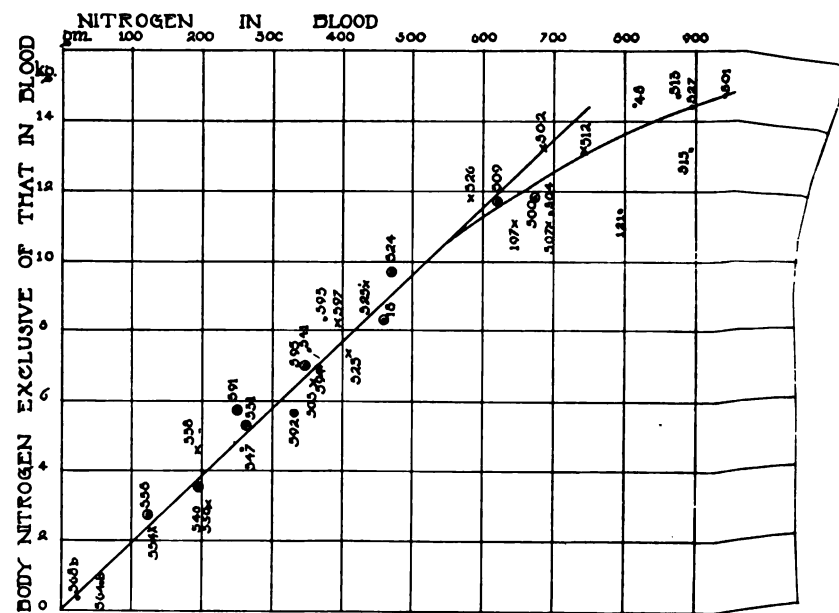
FIG. 14. Relation between total body nitrogen exclusive of that in the blood and nitrogen in the blood.

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in more blood. The same relation is shown when the nitrogen
in the blood is plotted against the body nitrogen (Fig. 14). All
the Group I animals 2 years old and over show a greater propor-
tion of total nitrogen in the blood to body nitrogen (exclusive of
that in the blood) than do the other animals.

The Relation of Blood to Body Surface.

It has been shown that the body surface is a power function
of the body weight and of the fat-free body weight, and that the
relation of blood to fat-free body weight is simple and fairly con-
stant. There should then be a relation between body surface
and total blood. Fig. 15 shows the values plotted on ordinary
cross section paper where a curve similar to the other power
functions is shown. Fig. 16 shows the values plotted on loga-
rithmic cross section paper. Here we find a straight line with
the inclination of $\frac{2}{3}$. When S is the surface area expressed in
square meters and B is the blood weight in kg., the formula is
$S = 0.8\,B^{\frac{2}{3}}$. The dotted lines were drawn to show a band which
would include practically all the points and thus aid in fixing the
general inclination.

SUMMARY.

A simple relation has been shown to exist between the total
body nitrogen and the fat-free empty weight of beef cattle. A
simple relation exists also between blood weight and fat-free
empty weight or between blood weight and total body nitrogen.

The surface area of beef cattle is a power function of the warm
empty weight, the formula for fat animals being $A = 0.154\,W^{\frac{2}{3}}$
and for other animals $A = 0.1186\,W^{\frac{2}{3}}$. The surface area is also
a power function of the fat-free empty weight. The formula for
all animals is $A = 0.1034\,W_1^{\frac{2}{3}}$.

The surface area is a power function of the total body nitrogen,
the formula being $S = N^{\frac{2}{3}}$.

The body surface and total blood are related in a similar manner,
the formula being $S = 0.8\,B^{\frac{2}{3}}$.
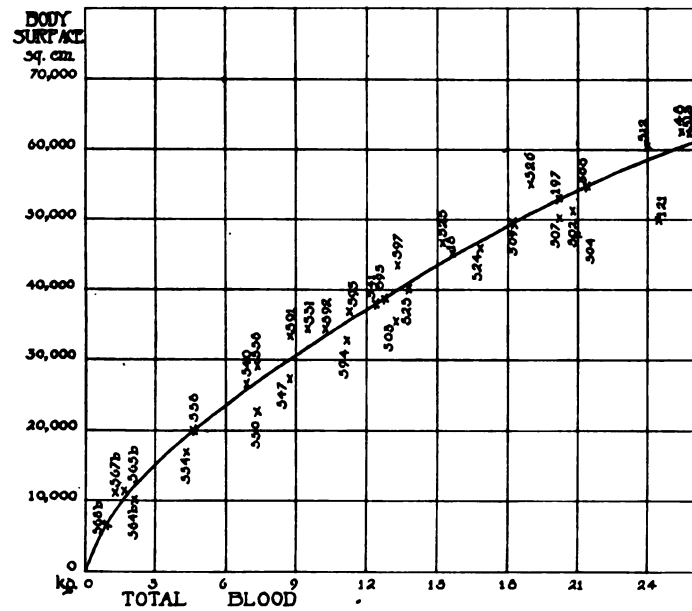
Fig. 15. Relation between body surface and blood weight.
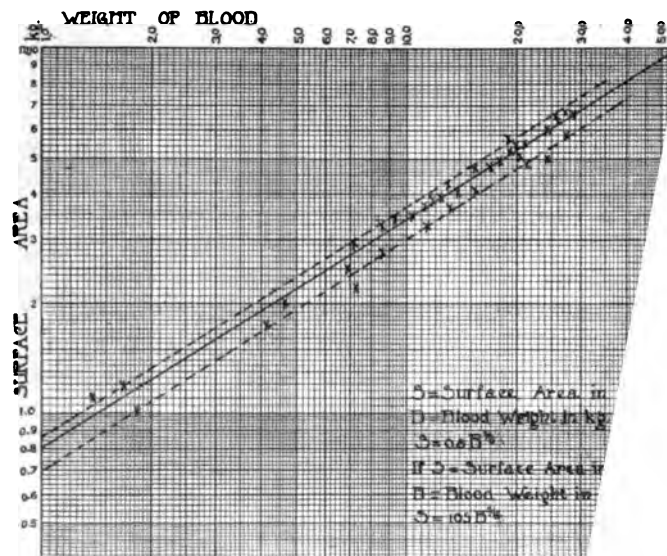
Fig. 16. Relation between body surface and blood
logarithmic cross section paper.

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CONCLUSIONS.

Since these relations have been shown to be fairly constant for animals under different conditions, it would seem to make little or no difference what unit of reference is used as a measure of basal metabolism as long as the unit used has been actually measured. It would seem also that for beef cattle the desired unit of reference could be calculated with considerable accuracy if the fatness of the animal and its condition with regard to fill can be determined. In many cases, however, the amount of fill in the beef animal can be disregarded. The author[1] has shown average variations in fill of 2 to 4 per cent of the live weight for mature animals of the three different groups above defined.

II.

In order to throw further light upon the question as to what unit of reference can best be used for basal metabolism and to test the validity of the conclusions given above, the maintenance data collected from a study of some of the animals used above càn be discussed.

In the Research Bulletin referred to above[1] the data are given in some detail for 19 different animals for a total of 27 maintenance trials. The maintenance periods of some of the animals resulted as a consequence of treatment given for a purpose other than the study of maintenance. For other animals the units of reference had to be calculated and also the consumption of digestible nutrients from data obtained upon other animals. In order to exclude variations due alone to these factors some of the animals will not be included in this study. Most of the animals were upon maintenance at a body weight or body condition different from that found at the time of slaughter. Table I shows what factors were used in calculating the various weights and measures used. For example, Steer 507 was slaughtered at the end of the maintenance trial and the units of reference for the maintenance data were actually measured values at the maintenance weight. For Steer 48 the digestion factors used were obtained from the animal itself while on maintenance. The other units were calculated from the percentages found for Steer 18 since these animals were very much alike. The surface area

TABLE I.

Animal No.	Digestion factors.	Empty weight.	Weight of nitrogen.	Weight of blood.	Surface area.	Weight at which data were taken.
500	Group III.	Own percentage.	Group III average.	Own percentage.	Own K.	62 kg. heavier.
500	" III.	" "	" III.	" "	" "	33 " "
502	Own.	Actual.	Own percentage.	" "	" "	50 " "
507	"	"	Actual.	Actual.	Actual.	Same weight.
509	"	Own percentage.	Own percentage.	Own percentage.	Own K.	50 kg. heavier.
512	Group II.	"	"	"	" "	65 " "
512	" II.	Actual.	Actual.	Actual.	Actual.	Same weight.
524	" III.	Own percentage.	Own percentage.	Own percentage.	Own K.	30 kg. heavier.
526	" II.	"	"	"	" "	87 " "
18	Own.	Actual.	Actual.	Actual.	Actual.	Same weight.
48	"	Percentage of No. 18.	Percentage of No. 18.	Percentage of No. 18.	Curve.	30 kg. heavier.
121	No. 18.	" " 18.	" " 18.	" " 18.	"	Weight same as No. 18
164	Own.	Own percentage.	No. 121.	Own percentage.	"	Double maintenance weight.
197	"	Actual.	Actual.	Actual.	Actual.	Same weight.
588	"	"	Group I, 2 yr.	Own	Curve.	"
589	588 and 197	Own percentage.	" I, 2 "	Own percentage.	"	134 kg. heavier.
596	Own.	Actual.	Actual.	Actual.	Actual.	Same weight.
597	No. 595.	"	"	"	"	"

ιs read off from Fig. 6, using the average curve for the Group
and Group III cattle.

Fig. 17 shows the total daily energy consumption for the dif-
ːent animals for body weight varying from 240 kg. to about 500
ː. It is seen that the energy consumption increases with the
·dy weight, but not proportionally, and there are large varia-
ιns. The points enclosed within circles indicate the animals
r which the digestion factors were assumed from data on other
ιimals.
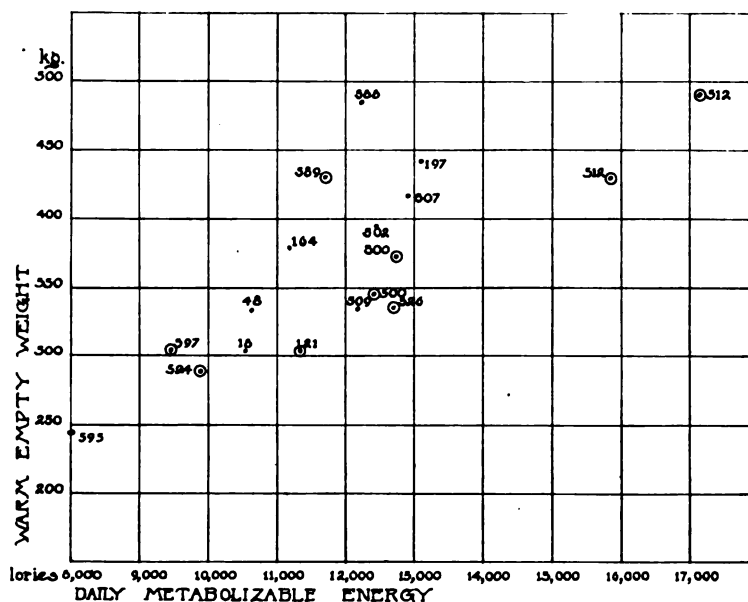
FIG. 17. Comparison of warm empty weight and total daily energy
nsumption for 16 beef cattle.

In Fig. 18 there is shown the heat consumption per kg. of body
ːight plotted against the body weight. The variations are great,
500 kg. steer consuming from 25 to 35 calories per kg. There
a marked tendency for the consumption of energy per kg. to
ːcrease with increasing weight. The variations from the aver-
ːe are 23.5 per cent.

In Fig. 19 are shown the values for heat consumption per square
eter of body surface plotted against body weight. A tendency
shown for the heat consumption per unit of surface area to in-
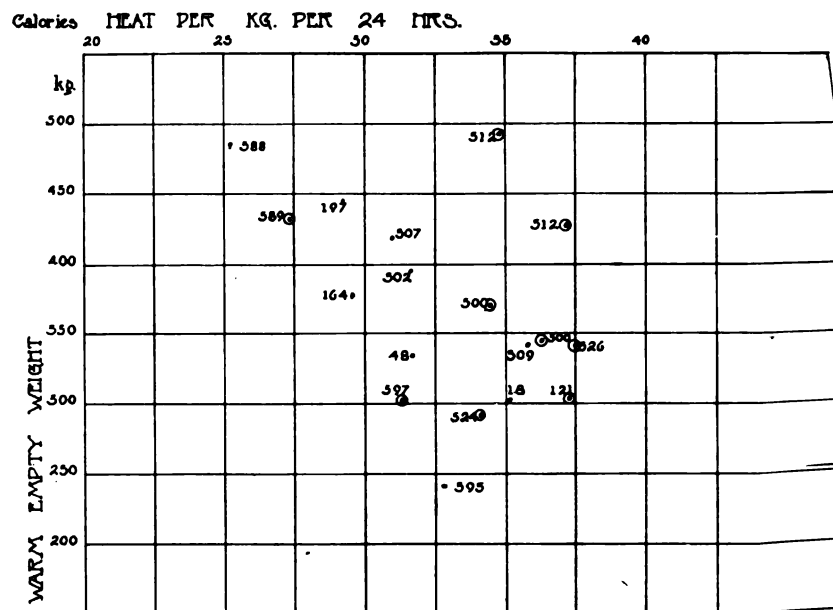
FIG. 18. Comparison of warm empty weight and daily heat consumption per kg. of body weight.
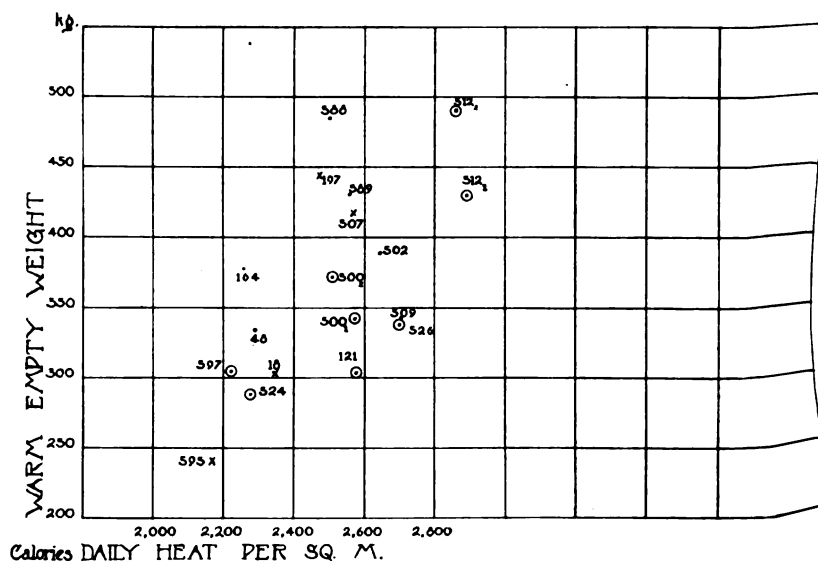
FIG. 19. Comparison of warm empty weight and daily heat consumption per square meter of body surface.
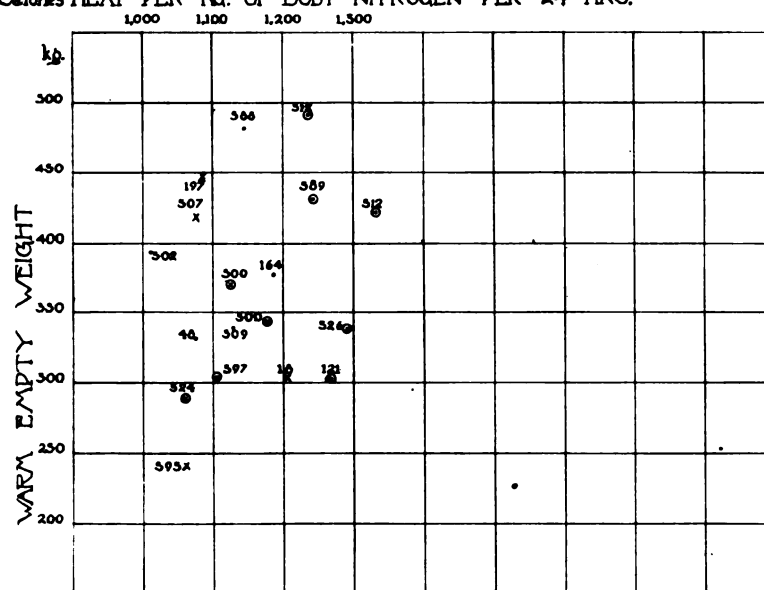
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FIG. 20. Comparison of warm empty weight and daily heat consumption per kg. of body nitrogen.
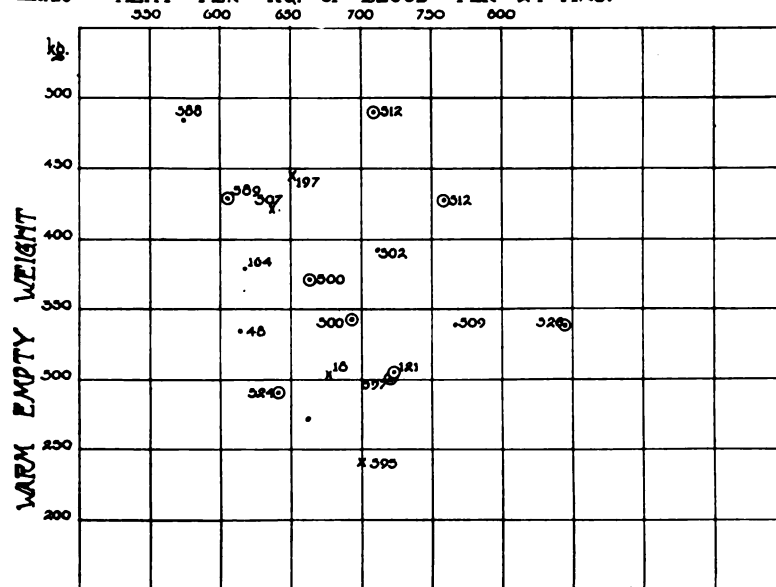
FIG. 21. Comparison of warm empty weight and daily heat consumption per kg. of blood.

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crease as the body weight increases. This has been shown[1] to be due to the greater weight sustained by the animal while standing and walking. The energy required for this is proportional to the weight sustained. The greatest variation from the average value in Fig. 19 is 15.6 per cent with one animal strikingly high. Omitting the values for this steer the variations are 11.3 per cent of the average. The points marked with a cross indicate the animals for which all the data were obtained under the conditions of maintenance.

Fig. 20 shows the heat consumption per kg. of body nitrogen. The points are distributed more nearly along a vertical line or vertical band. The extreme variation is 14 per cent of the average.

Eig. 21 shows the heat consumption per kg. of blood. The tendency is for a slight decrease in this consumption per unit of blood as the animal gets heavier. An extreme variation of 15 per cent from the average is shown. The steer at the extreme right seemed to be abnormal with regard to the percentage of blood in the body, and is therefore omitted.

Several factors have been shown to enter into the consideration of the cause of the variations shown above. These were discussed at length in the Bulletin already referred to. Since it can be assumed that they affected similarly the heat consumption per the different units of reference, the validity of the above comparisons should not be questioned. On this basis the smallest variations are shown in the heat consumption per unit of body surface and the greatest variations in the heat consumption per unit of body weight. Per unit of body nitrogen (protoplasmic tissue) and per unit of blood the variations are not much larger than per unit of body surface. If there is a choice of units it would seem to fall upon the surface area. Since this can easily be calculated from the body weight by the formulas proposed by the writer of this paper, it would seem that this unit fits well the needs of the investigator in animal nutrition, especially with beef cattle.

These conclusions are opposed to the views of Benedict[4] but are in agreement with those of Du Bois[5] and Graham Lusk.[6]

[4] Benedict, F. G., J. Biol. Chem., 1915, xx, 279.

[5] Du Bois, E. F., J. Am. Med. Assn., 1914, lxiii, 827. Du Bois, D., and Du Bois, E. F., Arch. Int. Med., 1915, xv, 868.

[6] Lusk, G., Science, 1915, xlii, 816.

THE ELECTROLYTIC DETERMINATION OF IODINE PRESENT IN ORGANIC MATTER.

By ROBERT B. KRAUSS.

(*From the Henry Phipps Institute of the University of Pennsylvania, Philadelphia.*)

In a previous paper[1] a method for the determination of the iodine present in tissues or other organic matter was described in detail. This method consists essentially of a fusion, reduction of any iodate formed, careful extraction of the iodides, precipitation with palladium chloride, and the estimation of the palladous iodide formed by colorimetric comparison with a standard solution of that substance.

This method has as a matter of practice been entirely satisfactory. It is far more sensitive than any method previously used and it is free from the objectionable features of several of the earlier procedures. As our immediate interest in the matter rested on the determination of iodine in tuberculous tissues,[2] obviously very complex, and in amounts frequently so small as to be barely perceptible by even this most sensitive method, it has seemed desirable to check the procedure in every possible way. I have therefore developed an electrolytic method of analysis to cover the same ground.

As the method already published involved the colorimetric determination of the palladous iodide formed in the reactions, so this further development has resolved itself into the problem of estimating the palladous iodide electrolytically. The amount of palladium and the amount of iodine are both determined, checking each other by calculation. This method is far less sensitive than the colorimetric determination. It can be used advantageously as a direct method of analysis if the amount of iodine

[1] Krauss, R. B., *J. Biol. Chem.*, 1915, xxii, 151.
[2] Lewis, P. A., and Krauss, R. B., *J. Biol. Chem.*, 1915, xxii, 159.

involved is relatively large, ± 0.0005 gm. After a series of determinations by the colorimetric method each involving amounts of iodine much smaller than this, it is advantageous to collect all the material and repeat the analysis electrolytically, thus making certain that there has been no gross error.

The following observations by previous workers bear on the problems involved in the electrolytic method.

Whitfield[3] estimated iodine electrolytically proceeding from the silver salt, weighing the metal after electrolysis. Vortmann[4] has developed an electrolytic scheme for the estimation of pure potassium iodide. Specketer[5] estimates iodine by electrolytic methods, using graded potentials.

None of these methods are suitable for routine determinations. The quantities of chlorides and bromides present interfere with the first two; the low and absolutely constant voltage as well as the necessity for absolute exclusion of air and definite acid concentration makes the last unsuitable.

That palladium can be deposited from a solution of palladammonium chloride was found by Smith[6] and Keller.[7]

It is well known that palladous iodide affords a separation of iodides from chlorides and bromides, and that it is insoluble in water, alcohol, ether, and dilute hydrochloric acid.

Method.

The substance to be analyzed is fused and treated exactly as in the colorimetric method until the alcohol-acetone solution of iodide is obtained. This is evaporated to dryness, taken up in 100 cc. of water, and an excess of palladous chloride added. After the addition of one or two drops of dilute hydrochloric acid the whole is heated to boiling in a beaker.

The black palladous iodide is filtered off, using a vacuum filtering dome, an ordinary funnel, and hardened filter paper.

It is not necessary to remove all the iodide from the beaker, but after washing the beaker and the filter with water, the beaker is used as a receiver and the iodide on the filter dissolved with hot aqueous ammonia into the beaker.

[3] Whitfield, J. E., *Am. Chem. J.*, 1886, viii, 421.

[4] Vortmann, *Z. Electrochem.*, 1895-96, ii, 314.

[5] Specketer, H., *Z. Electrochem.*, 1897-98, iv, 539.

[6] Smith, E. F., *Am. Chem. J.*, 1891, xiii, 206; 1892, xiv, 435.

[7] Smith, E. F., and Keller, H. F., *Am. Chem. J.*, 1890, xii, 212.

All the palladous iodide remaining in the beaker is carefully dissolved. For small weights of iodide the solution is electrolyzed directly; for larger ones it is first made up to a definite volume in a flask and then an aliquot part electrolyzed.

With a weighed gauze platinum cathode, the solution is electrolyzed in a platinum dish at a dilution of 60 cc. and at 65°C. About 1 cc. of ammonia is added. The current may vary from 0.5 to 1 ampere, the voltage from 5 to 10 volts. Rotating the cathode at about 300 R. P. M., the palladium is readily deposited.

The current is interrupted, the cathode carefully washed with water over the dish, then after removal of the cathode from the spindle of the rotator, it is washed with water, alcohol, and ether. After warming in an electric oven and cooling in a desiccator, it is weighed. About 25 minutes are required for the complete deposition.

The solution is evaporated to half volume, with the addition of 1 cc. of 10 per cent sodium hydroxide and 1 gm. of sodium potassium tartrate, and is covered with a watch glass.

After cooling and diluting to 40 to 50 cc., the iodine is deposited on the silver electrode as anode, the current having been reversed. The pressure should be 2 volts, current 0.01 to 0.05 amperes, and the anode stationary. Time required, 30 to 40 minutes. Stirring at this point seems to favor the detachment of silver iodide. After washing as above, the electrode is dried at 300°C. in an electric oven. It is cooled and weighed.

All reagents must be carefully examined for chlorides, and the silver is best purified by electrolysis.

Preparation of Electrodes.

The silver plated anode is prepared as follows: A circular platinum gauze electrode is heavily plated from a silver ammonium oxalate solution, using 1 to 2 amperes and 5 to 10 volts and rotating at about 300 R. P. M. This electrode is washed with ammonia water, distilled water, and ignited. It is then boiled with hot water for half an hour, washed in water, absolute alcohol, absolute ether, dried at 300°C. in an electric oven, and weighed.

After each determination the silver iodide is removed by cyanide and the washing repeated. An electrode lasts from six to twelve determinations before replating is necessary.

The platinum cathode (circular, gauze) is freed from palladium by dipping into a mixture of saturated salt solution and chromic acid. This scarcely attacks the platinum.

The following tables show values obtained by this method.

In Table I are the figures obtained by electrolyzing a portion of a standard solution of palladammonium iodide prepared from a standard solution of potassium iodide.

Table II shows values obtained by use of the complete method on some typical organic iodine compounds.

Table III shows a check on a series of determinations by the colorimetric method of the iodine content of tuberculous and normal tissues.

TABLE I.

	Iodine.	Palladium.
	mg.	*mg.*
Present....................	2.50	1.054
Found.....................	2.60	1.05
"	2.45	1.08
"	2.45	1.00

TABLE II.

Iodobenzoic acid 0.5 gm. taken. 51.21 per cent I. Dilution 500 cc.	Iodine.		Palladium.	
	Present.	Found.	Present.	Found.
cc.	*mg.*	*mg.*	*mg.*	*mg.*
1	0.512	0.50	0.216	0.20
5	2.56	2.50	1.08	1.10
10	5.12	5.08	2.16	2.11
Iodoacetanilide 0.5 gm. 48.65 per cent I. Dilution 500 cc.				
1	0.49	0.46	0.21	0.22
5	2.43	2.40	1.03	1.00
10	4.86	4.84	2.05	2.05
Diododiphenyl 0.5 gm. 62.56 per cent I. Dilution 500 cc.				
1	0.63	0.64	0.27	0.26
5	3.13	3.10	1.32	1.30
10	6.26	6.21	2.64	· 2.63

TABLE III.

Check on Twenty-two Colorimetric Determinations.

	Iodine.	Palladium.
	mg.	*mg.*
Colorimetric..................	7.016	Calculated..... 2.96
Electrolytic..................	6.90	Found......... 2.82

SUMMARY.

1. The electrolytic method may be used to estimate gravimetrically the iodine present in organic tissues or in organic iodides.

2. It provides an adequate check on a series of determinations by the palladous iodide colorimetric method.

3. In all cases the estimation of both cation and anion gives another check.

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STUDIES ON THE THEORY OF DIABETES.

VI. THE BEHAVIOR OF *dl*-GLYCERIC ALDEHYDE IN THE NORMAL AND DIABETIC ORGANISM.

BY W. D. SANSUM AND R. T. WOODYATT.

(*From the Otho S. A. Sprague Memorial Institute Laboratory for Clinical Research, Rush Medical College, Chicago.*)

Pure crystalline *dl*-glyceric aldehyde was administered by different routes to normal rabbits and guinea pigs and to completely phlorhizinized and glycogen-free dogs. The experiments continue an investigation reported 5 years ago,[1] and are probably the first in which the pure aldo triose has been studied in living animals. The results are discussed in their relation to the theory of metabolism and diabetes, which forms the theme of the present series of communications. Coincident with the work of establishing the behavior of *dl*-glyceric aldehyde in the diabetic organism, further observations were made upon the technique of deglycogenation by means of epinephrin, which was described in our last paper.[2] Epinephrin was given as before once every 3 hours subcutaneously, but the dosage was varied in different experiments. Further experimentation with different dosages in a series of experiments not here reported taken in conjunction with the present series lead to the conclusion that 0.04 mg. of epinephrin per kilo of body weight subcutaneously once every 3 hours nearly always suffices to eliminate all glycogen within the first 24 hours of the phlorhizin régime and exerts little if any harmful effect upon the animal. With smaller doses the time necessary for deglycogenation is unduly protracted, while larger ones—although capable of discharging the glycogen in 12 to 18 hours—are unnecessarily severe and frequently exert deleterious effects on the circulation and secretions.

[1] Woodyatt, R. T., *J. Am. Med. Assn.*, 1910, lv, 2109.

[2] Sansum, W. D., and Woodyatt, R. T., *J. Biol. Chem.*, 1915, xxi, 1.

Further studies were also made of the character of the urinary retentions which so frequently develop in phlorhizinized dogs after the administration of certain classes of chemical substances, as dealt with in our paper on narcotic drugs.[2] The urinary suppressions which followed the administration of glyceric aldehyde were at first so marked that the urine gave no conclusive proof that the triose had been converted into glucose. But it was found that the suppressions could be minimized or annulled by an appropriate alkaline diuretic therapy based on the principle that the retentions were due to acid in the tissues, secondary to tissue asphyxia and provocative of processes akin to edema. It was then demonstrated that glyceric aldehyde may be converted almost quantitatively into glucose. The control of suppressions by the use of alkali and a diuretic sålt solution supports the interpretations which were made in our last paper that the decreases in the glucose, nitrogen, and acetone body eliminations which were observed after the administration of ether, chloroform, and acetaldehyde were expression of retentions rather than primary metabolic changes. The effects of the alkaline diuretic treatment upon the suppressions which follow the administration of glyceric aldehyde suggest that other toxic retentions might be similarly managed and that by a suitable supplemental treatment of the animals the phlorhizin technique may still be used for testing the behavior of many substances which could not otherwise be given without so interfering with elimination as to make the results of urine analysis valueless. It is also considered that these results of therapy directed against toxic retentions in diabetic dogs have a certain clinical bearing.

HISTORICAL.

The chemical literature concerning glyceric aldehyde with particular reference to the technique of its preparation has been collected and enlarged in two articles by E. J. Witzemann[3] of this laboratory. The following points may be repeated, since they aid in the criticism of the biological experiments. Glyceric aldehyde was first isolated in pure form in 1898 by Wohl[4] by means of an elaborate synthetic method which, until 1914, remained the only means by which pure glyceric aldehyde could be

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[3] Witzemann, E. J., *J. Am. Chem. Soc.*, 1914, xxxvi, 1908, 2223.

[4] Wohl, A., *Ber. chem. Ges.*, 1898, xxxi, 1796, 2394.

prepared. Before 1898 the actual properties of this triose were therefore not established, although as early as 1860 some of the more important had been prophesied correctly. In 1887 Grimaux, and Fischer and Tafel established the existence of trioses in syrups obtained by the oxidation of glycerol; and in 1897 Fenton and Jackson proved that much glyceric aldehyde was present in syrups left by the oxidation of glycerol with H_2O_2 in the presence of ferrous sulphate, but no crystalline glyceric aldehyde was ever separated from such syrups until this was accomplished by Witzemann in 1914.

With the growth of chemical interest in the trioses as hypothetical substances they began to be assigned rôles in schemes of animal sugar metabolism and particularly the aldo triose. But glyceric aldehyde has never yet been demonstrated in a normal body fluid or tissue, although this has been claimed erroneously on several occasions, and until 1904, no direct experiments had been made to establish its actual physiological properties. This was doubtless because the one known method of preparing it was and is time-consuming and difficult to a degree which makes its successful execution a chemical feat.

In 1904 Neuberg[5] stated that experiments had been made in conjunction with Blumenthal in which crude "glycerose," prepared by the oxidation of glycerol with H_2O_2 in the presence of a ferrous salt (Fenton and Jackson's method) had been administered to normal and diabetic men and animals. According to Neuberg, the glycerose was a "sweet colorless syrup" and "wholly non-toxic." When given to normal animals and men by different routes it was "completely oxidized even in very large doses." It was found to be a glycogen builder and the tolerance limit for it was "greater than for all artificial and most natural sugars including glucose." "Remarkable" was "the behavior of glycerose in the diabetic organism. . . ." "Here also it" was "completely burned without increasing the existent glycosuria in the least degree." As recently as 1913 Neuberg[6] has cited this report without reference to subsequent communications, from which it would appear that these statements have not been supplemented by a detailed description of experiments. They record, however, the first effort to test experimentally the effects of glyceric aldehyde in the body.

In 1910 one of us[1] published the results of experiments made with similar syrups. These syrups were faintly colored and sweet, with an aromatic flavor and reducing power equivalent to 50 to 60 per cent glucose solutions. Subsequent work has proved that they contain a large quantity of dl-glyceric aldehyde, which Witzemann succeeded in separating as the diethyl acetal and finally crystallizing, but since pure glyceric aldehyde is found to have the same reducing power as glucose, the total content of glyceric aldehyde in these syrups was never above 60 per cent. They were found not to be wholly non-toxic, as Neuberg and Blumenthal had held for theirs; and it has since been shown that crystalline dl-glyceric aldehyde is three

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[5] Neuberg, C., Arch. Anat. u. Physiol., Physiol. Abt., 1904, 571.

[6] Neuberg, Oppenheimer's Handb. Biochem., 1913, 588.

times as toxic as glucose when given by mouth and six to eight tir
toxic by parenteral routes. It was also noted that syrups of thir
when fed to cases of severe diabetes in dilute subdivided doses (
increases of glycosuria sometimes amounting roughly to upwards
per cent of the total weight of the syrup given. In these cases the
no appreciable effect on the excretion of ammonia and acetone !
(Table I). The essential features of this observation have sinc
confirmed with pure material. It was observed, however, that whi
glycerose was given in relatively concentrated solutions and all i
time, there was little immediate increase of the glycosuria and ir
cases there was a remarkable decrease in the output of ammoni

TABLE I.

*Abstract from the Protocol of a Case of Human Diabetes on a Constar
(January, 1909). On January 25, 26, and 27 the Patient Slowly Si)
Daily 40 Gm. of Glycerose Syrup in Dilute Solution Throughout t
Waking Day, Thus Receiving 120 Gm. in 3 Days. The Periods
Are of 24 Hours Each. The Unknown Glycogen Content
Naturally Constituted a Source of Errcr.*

Date.	Urine volume.	Glucose.	β-Hydroxy-butyric acid.	Acetone.	Ammonia.	Nitrogen.	Remarks.
1909	cc.	gm.	gm.	gm.	gm.	gm.	
Jan. 24	2,475	60.1	23.8	4.0	1.7	18	
" 25	2,910	96.9	19.5	3.9	1.7	18	3 glyceric aldehyde day: which together with tl days the average glu cretion was 32 gm. at average for 5 days pr this increase corres: roughly to 80 per cen glycerose given.
" 26	2,700	110.5	18.0	3.9	1.6	18	
" 27	2,690	107.6	18.7	4.0	1.4	16	
" 28	3,310	85.0	25.0	4.7	1.9	18	
" 29	3,340	80.0	26.0	4.7	2.0	18	
" 30	2,800	72.0	23.8	4.2	1.9	18	

acetone bodies. An example of such an effect is shown in Table I
this time the idea was entertained that the more concentrated and
dosages, producing higher concentrations of glyceric aldehyde in tl
sues, favored direct burning and concomitant antiketogenesis, whi
slow administrations, producing lower concentrations of glyceric ald
in the tissues, had permitted larger percentages to be transforme
glucose and so to escape oxidation. Such results appeared more i
mony with Neuberg's view. Later work would indicate, however, th
failures to obtain more marked increases of glycosuria after the
doses of glycerosé were due at least in part to retentions induced l
toxic action of the glycerose on the kidneys and other tissues and th

striking suppressions of the acetone body and nitrogen outputs were at least in part simultaneous expressions of the same process. These apparently favorable effects were accordingly questioned.[7]

In 1912 reports of experiments with glyceric aldehyde appeared from several sources. Parnas[8] perfused tortoise livers with solutions of dl-glyceric aldehyde syrups which he prepared in accordance with the method of Wohl. He found that if the perfusion fluid contained sufficient oxygen in addition to dl-glyceric aldehyde, glycogen was formed, but not otherwise. Smedley[9] added impure glyceric aldehyde solution to liver emulsions and noted that the triose disappeared without evidences of a new

TABLE II.

An Earlier Experiment on the Same Case. On January 19 the Patient Received by Mouth 75 Gm. of Glycerose Syrup in Lemonade, Which He Drank Slowly in the Course of an Hour.

Date.	Urine volume.	Glucose.	β-Hydroxy-butyric acid.	Acetone.	Ammonia.	Nitrogen.	Remarks.
1909	cc.	gm.	gm.	gm.	gm.	gm.	
Jan. 10–15	2,550	68	23	4.0	1.4	16	
" 16	2,170	65	19	3.8	1.3	17	
" 17	2,630	74	26	4.2	1.3	18	
" 18	2,650	72	22	3.9	•1.6	19	
" 19	2,500	87	5.3	1.6	0.8	17	Glyceric aldehyde day with gross glucose increase of 17 gm. above the average of 8 preceding days. This corresponds to about 23 per cent of the weight of the syrup given.
" 20	2,360	54	24	3.5	1.1	17	
" 21	2,620	74	20	4.2	1.6	17	
" 22	2,850	74	25	4.6	1.8	17	
" 23	2,475	60	24	4.00	1.9	18	

[7] This same interpretation of a delayed urinary excretion of sugar and the acetone bodies as an improvement of the diabetic metabolism, still recurs in the literature with remarkable frequency. It has been made by others with reference to the dicarboxylic acids, aldehydes, transfusions of blood, injections of various tissue extracts, and we think also with reference to alcohol, the von Noorden oatmeal cure, and other diets which can cause edemas. Neubauer raised this point with reference to the glycerose experiments in 1910, also Edsall, and we have frequently reiterated the fact that the results of urine analysis even when taken in conjunction with blood analyses are not necessarily a true reflection of the metabolic status.

[8] Parnas, J., *Zentr. Physiol.*, 1912, xxvi, 671.

[9] Smedley, I., *Jour. Physiol.*, 1912–13, xliv, 203.

formation of glycogen. Since under the conditions of these experiments
there would necessarily have been a lack of oxygen, her results are in
harmony with those of Parnas. Later Embden[10] and his coworkers, for
the first time employing a pure although uncrystallized dl-glyceric alde-
hyde prepared by Wohl's synthesis, studied its behavior in surviving tis-
sues (liver and washed blood corpuscles apparently under conditions of
relative asphyxia) with particular reference to its convertibility into lactic
acid. It was found convertible into lactic acid under these conditions,
and on the basis of these experiments Embden has constructed a scheme
of sugar metabolism in which he makes a cleavage of the glucose molecule
into optically active glyceric aldehyde a major step in the normal glucose
breakdown.

In summarizing the experimental work up to the present time.
the following facts appear to have been demonstrated: (1) Sur-
viving tissues (with a deficient oxygen supply) cause the disap-
pearance of dl-glyceric aldehyde added to or perfused through
them, and at least a part of the triose is then converted into lactic
acid. (2) The diabetic organism may convert dl-glyceric alde-
hyde into d-glucose. (3) Surviving livers perfused with oxygen-
ated solution of dl-glyceric aldehyde may convert a part of the
latter into glycogen. (4) When administered to normal animals
and men, considerable amounts of glyceric aldehyde disappear
and are probably utilized.

New Experiments with dl-Glyceric Aldehyde.

Material.—As a basis for these experiments we have used
crystalline dl-glyceric aldehyde prepared by E. J. Witzemann
from glyceric aldehyde diethyl acetal, which was obtained either
by means of Wohl's synthesis, or more directly from glycerol
oxidation mixtures as described in the two papers quoted above.[3]
The material is a stable crystalline powder which is non-hygro-
scopic and dissolves slowly in water. It represents dimolecular
crystals and is tasteless. It is also odorless, but after standing
for days in a stoppered bottle a faint flavor of burnt sugar is
noticeable. The crystals melt at 142°C., or 4° higher than any

[10] Embden, G., Kalberlah, F., and Engel, H., *Biochem. Z.*, 1912, xlv,
45. Kraske, B., 81. Kondo, K., 88. Von Noorden, K., jun., 94. Embden,
G., Baldes, K., and Schmitz, E., 108. Embden, G., and Oppenheimer,
M., 186.

previously recorded preparation. (138°, Wohl and Neuberg.[11]) Aqueous solutions of this material are clear and colorless, and unlike the dimolecular crystals, have a mild sweet taste. They reduce Fehling's solution in 1 to 5 minutes at room temperature, give a vivid aldehyde reaction with Schiff's reagent, and show all the characteristic reactions which have been described for dl-glyceric aldehyde. Observations made of its reducing power show that dl-glyceric aldehyde corresponds gram for gram with glucose. For the *detection of glyceric aldehyde in a body fluid* use has been made of the following properties: (1) Its power to reduce Fehling's solution at room temperature within 3 minutes or immediately at 50°C. (2) Its power to combine with phenylhydrazine acetate to yield insoluble crystals of glyceric aldehyde phenylosazone (m. p. 132°C.) slowly at room temperature, more rapidly at 50°C. (3) Its ability even in minute traces to produce a slowly deepening, finally vivid violet coloration when added to a solution of fuchsine decolorized with H_2SO_3 and freed of excess of the acid (Schiff's aldehyde test).

Toxicity and Tolerance.

Tests were made with rabbits and guinea pigs. The triose was administered in 2 to 7 per cent aqueous solutions subcutaneously and into the stomach. *When given by the stomach*, doses up to 2.8 gm. per kilo of body weight caused no apparent symptoms or qualitative urinary changes, although there was some diminution of the flow. A dose of 5 gm. per kilo (a rabbit of 1,400 gm. receiving 100 cc. of 7 per cent solution by stomach tube) was followed immediately by *diarrhea with free glyceric aldehyde in the passages*, a marked diminution in the rate of urination, but no melituria. *Thereafter for 10 days the urine contained albumin.* A dose of 6.8 gm. per kilo caused death in a guinea pig within 4 hours. There was no catharsis and no melituria was demonstrated. The animal died quietly, and at autopsy the right heart was filled with blood, the abdominal viscera showing passive congestion with only slight parenchymatous swelling and cloudiness, most evident in the kidneys. *Subcutaneously*, doses of 2.2 and 2.4 gm. per kilo given respectively to a rabbit and a guinea pig

[11] Wohl and Neuberg, *Ber. chem. Ges.*, 1900, xxxiii, 3095.

caused complete suppressions of urine and death, with no cathar-
sis, and with autopsy findings similar to those mentioned above.
Doses of 1 gm. per kilo were borne with no apparent symptoms.

To sum up: The average lethal dose by the subcutaneous route
appears to have been about 2.2 gm. per kilo; by the alimentary route
between 5 and 6 gm. per kilo. After as much as 1 gm. per kilo
subcutaneously and 3 gm. per kilo by stomach no unfavorable
symptoms were noted. Parallel experiments in the same set of
animals showed for d-glucose given by either route a minimum
lethal dose of 18 gm. per kilo, indicating a toxicity for this prepa-
ration of dl-glyceric aldehyde about three times that of glucose
by stomach and eight times as great subcutaneously. Glyceric
aldehyde was not found in the urine after oral or subcutaneous
doses.[12]

Behavior of dl-Glyceric Aldehyde in Phlorhizin Diabetes.

Dogs weighing 10 to 15 kilos were given 1 gm. of phlorhizin
(Merck) triturated in a mortar with about 20 cc. of olive oil once
every 24 hours. Deglycogenation was effected by 3 hourly
subcutaneous doses of (1: 1,000) adrenalin solution (Parke, Davis
and Co.). Some of the experiments antedate the establishment
of the routine which was later adopted, so that sometimes more
and sometimes less than the usual 0.04 mg. of epinephrin per kilo
was given, but the protocols are reproduced in sufficient detail
to prove that deglycogenation was in every case satisfactory.
All details as to the collections of urine and analytical methods
were as described in the paper on narcotics.[2] Attempts were
first made to administer the material by mouth. But although
the diabetic dogs drank solutions spontaneously, the material
was twice vomited, and owing to the cost of the preparation we
did not risk a further loss. Subsequently it was given subcu-
taneously or by vein.[13]

[12] For the behavior of glyceric aldehyde when given by vein, see the
following article.

[13] Dakin, H. D. (*J. Biol. Chem.*, 1913, xiv, 328), has warned against
intravenous administrations on the ground that they may cause extra
sugar to appear from sources other than the substance injected. This
was apparently based on observations upon intravenous administrations
in dogs prepared by simple phlorhizination with no supplemental deglyco-

The experiments with phlorhizinized dogs fall into two groups, A and B.

In Group A (Experiments I, II, III, and IV) glyceric aldehyde was given in 10 to 15 gm. single or subdivided doses with no supplemental treatment, to dogs weighing 13 to 17 kilos. All the animals died within 18 hours, death being preceded by marked urinary retentions which became noticeable within the first 6 hours but were more marked in the after-periods. In two experiments (I and II) no extra sugar appeared. In two (III and IV) the extra sugar was 4.49 and 3.95 gm., with gross increases of 2.46 and 2.63 gm. respectively. Thus in two of four experiments there were absolute and relative glucose increases corresponding respectively to 30 and 40 per cent of the weight of triose administered. These dogs had received epinephrin until it could be stopped for 6 hours and again given in the next 6 hours without changing the G: N ratio, so that the extra sugar which appeared could not have been derived from glycogen. It is accordingly attributed to the glyceric aldehyde, but it was not sufficient in amount to indicate a quantitative conversion. Reference to the protocol of Experiment IV will show that in the first after-period extra sugar reckoned in the usual way was 2.77 gm. But in this period there were marked absolute decreases in all the secretions, indicative of disturbed elimination, and under these conditions it is unsafe to interpret a rising G: N ratio as evidence of gluconeogenesis, especially when delayed.

In Group B (Experiments V, VI, and VII) the dosage of glyceric aldehyde was reduced to 5 gm., to lessen the toxic effects and urinary retentions. These dogs also received supplemental treatments consisting of intravenous injections of an alkaline diuretic salt solution. All the animals lived. In every case there was a marked gross increase of the urinary glucose and, except in Experiment VII, a corresponding output of extra sugar. The extra sugar output, following 5 gm. doses of glyceric aldehyde, were in Experiment V, 4.92 gm. (certified by a gross increase of 5.21 gm.), and in Experiment VI, 4.84 gm. (with a gross increase of 4.85 gm.). Thus the 10 gm. of glyceric aldehyde

TABLE III.

Experiments I, II, III, and IV. dl-Glyceric Aldehyde, Designated in Text as Group A, with No Alkaline Diuretic Treatment. Suppressions Marked in All. Definite Evidences of New Sugar in Only Two, and Moderate in These (III, IV).

Experiment No.	6 hr. period.	Glucose.	Nitrogen.	G:N	Extra sugar.†	Gross sugar increase.	Acetone.*	β-Hydroxy-butyric acid.	Total** acetone bodies.	Remarks.
		gm.	gm.		gm.	gm.	gm.	gm.	gm.	
I	1–9									Animal received ostensibly 0.1 mg. epinephrin per kilo of body weight subcutaneously throughout the experiment. Unduly long preparatory period of 72 hours, owing to deterioration of the epinephrin solution, the real dosage being much smaller than intended.
	10	24.94	3.39	7.36						
	11	14.44	3.24	4.44						
	12	14.81	4.10	3.61						
	13	13.38	4.35	3.07						
	14	12.31	4.10	3.00						
	15	11.50	3.61	3.18	0.0	0.0				Glyceric aldehyde 10 gm. in 133 cc. H₂O subcutaneously in Period 15 followed by retentions. Death in Period 17.
	16	1.95	0.70	2.80						
	17									
II	1–3									Epinephrin 0.1 mg. per kilo of body weight every 3 hours to end of Period 5. Deglycogenation complete in 18 hours but this dosage is unduly severe.
	4	6.19	2.16	2.86						
	5	7.56	2.77	2.73						
	6	7.06	2.63	2.68	0.0	0.0				Glyceric aldehyde 10 gm. in 500 cc. H₂O subcutaneously at the rate of about 42 cc. every 30 minutes in Period 6.
	7	4.53	1.26	3.60	1.13	0.0				
	8	1.15	0.39	2.95						Retentions. Death in Period 9.
	9									
III	1–4									Epinephrin 0.033 mg. per kilo, every 3 hours to Period 12. G:N virtually constant after 36 hours.
	5–6	13.75	4.19	3.28						
	7–8	12.95	4.03	3.21						
	9–10	13.78	4.58	3.00						
	11–12	12.28	4.19	2.93			0.428	0.289	1.056	
	13	11.72	3.76	3.11						Epinephrin omitted in Period 13.
	14	11.41	3.91	2.92			0.391	0.340	1.041	Epinephrin resumed in Period 14. Dog glycogen-free.
	15	14.03	3.17	4.42	4.49	2.46	0.212	0.293	0.673	Glyceric aldehyde 15 gm. in 200 cc. H₂O subcutaneously in Period 15. Death in Period 16.
	16									

TABLE III—*Continued.*

Experiment No.	6 hr. period.	Glucose.	Nitrogen.	G: N	Extra sugar.†	Gross sugar increase.‡	Acetone.*	β-Hydroxy-butyric acid.	Total** ace-tone bodies.	Remarks.
		gm.	*gm.*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
I V	1-4									Epinephrin ostensibly 0.1 mg. per kilo every 3 hours until end of Period 7.
	5	12.60	4.23	2.97						
	6	12.39	4.12	3.01						Deglycogenation in 18 hours with no injury, but the adrenalin had lost strength, and the actual dose was smaller.
	7	11.31	3.66	3.08			0.077	0.309	0.446	
	8	11.88	3.89	3.06			0.067	0.370	0.490	No epinephrin.
	9	10.06	3.28	3.06			0.056	0.296	0.396	Epinephrin resumed. Dog proved glycogen-free.
	10	13.60	3.16	4.30	3.95	2.63	0.025	0.237	0.281	Glyceric aldehyde 10 gm. subcutaneously. Epinephrin continued.
	11	7.70	1.61	4.08	2.77	0.0				Epinephrin as before.
	12									Death.

† Extra sugar = G − (N × G: N), when G = gm. glucose for a period, N = gm. nitrogen for the same period, and G: N = the average ratio for the preceding 12 hours.

‡ Gross sugar increase reckoned as gm. glucose in a given period minus the average gm. glucose for 12 hours preceding.

* Inclusive of acetoacetic acid.

** Reckoned in terms of β-hydroxybutyric acid.

given in Experiments V and VI gave rise to an output of 9.76 gm. of extra sugar, with a gross sugar increase of 10.06 gm., which clearly demonstrates a practically complete conversion into glucose of all the glyceric aldehyde administered. In Experiment VII extra sugar was only 1.46 gm.

In all the experiments of Group B evidences of retention were missing or moderate. Even in the earlier experiments, I to IV, they had been less marked in the experimental periods than subsequently, but in Experiments V to VII they were absent in the experiment periods. This difference is attributed to the more moderate dosage of glyceric aldehyde and to the treatment given. That the decreased dosage was not alone responsible for the difference and that the treatment also played an important part is demonstrated by the fact that in Experiments V and VI treatment was discontinued in the after-periods and in these periods signs of retention supervened, although less markedly than in the

experiments of Group A, whereas in Experiment VII, the alkaline diuretic treatment was continued into the after-periods, with the result that no retentions occurred at all.   On the contrary, in this case both the glucose and nitrogen at the end of 18 hours were actually higher than they had been during the whole experiment, an occurrence which distinguishes this from all the

TABLE IV.

Experiments V, VI, and VII.   dl-Glyceric Aldehyde.   Experiments Designated in Text as Group B, with Alkaline Diuretic Treatment. Suppressions Moderate in Two, Negative in One.   Gluconeogenesis Definite in All and Virtually Complete in Two (V, VI).

Experiment No.	6 hr. period.	Glucose.	Nitrogen.	G : N	Extra sugar.	Gross sugar increase.	Remarks.
		gm.	gm.		gm.	gm.	
V	1–4						Epinephrin 0.025 mg. per kilo of body weight subcutaneously every 3 hours.
	5	18.38	4.05	4.53			G : N still high after 30 hours, owing to small dosage.
	6						Epinephrin continued and analyses omitted.
	7	13.75	4.84	2.84			Epinephrin continued; low ratio and apparent deglycogenation after 36 hours.
	8	13.25	4.40	3.01			Epinephrin omitted, with no significant change in figures.
	9	15.00	4.47	3.35	1.90	1.50	Epinephrin resumed; shows trace of glycogen was present.
	10	11.75	4.41	2.66			No epinephrin. Alkaline diuretic (Fischer's solution) 20 cc. by vein.
	11	12.13	4.20	2.89			No treatment.
	12	15.78	3.29	4.03	4.92	4.84	Glyceric aldehyde 5 gm. (5 per cent solution), also 20 cc. Fischer's solution by vein.
	13	9.56	3.74	2.55			No treatment.
	14	9.25	3.61	2.56			No treatment. Dog strong and later recovered.

TABLE IV—*Continued.*

Experiment No.	6 hr. period.	Glucose.	Nitrogen.	G : N	Extra sugar.	Gross sugar increase.	Remarks.
		gm.	*gm.*		*gm.*	*gm.*	
VI	1–4						Epinephrin 0.025 mg. per kilo of body weight subcutaneously every 3 hours.
	5	12.63	3.05	4.14			Same. Ratio still high owing to small epinephrin dosage.
	6	11.69	3.58	3.26			Epinephrin continued.
	7	11.87	3.86	3.08			Epinephrin continued.
	8	10.50	3.61	2.90			No epinephrin.
	9	12.19	3.95	3.10	1.38	1.51	Epinephrin resumed and shows only trace of glycogen.
	10	11.81	4.25	2.77			Fischer's solution 20 cc. by vein increases nitrogen but not sugar.
	11	11.06	3.86	2.84			No Fischer's solution. No epinephrin.
	12	11.13	4.13	2.70			Fischer's solution 20 cc. by vein increases nitrogen but not sugar.
	13	11.56	3.92	2.94			No treatment.
	14	16.56	4.17	3.97	4.84	5.21	Glyceric aldehyde 5 gm. (in 5 per cent solution) subcutaneously, 20 cc. Fischer's solution by vein.
	15	9.75	2.62	3.72			No treatment. Retentions moderate. Dog recovered.
VII	1–4						0.03 mg. epinephrin per kilo of body weight every 3 hours.
	5	17.06	4.41	3.86			Epinephrin continued.
	6	15.00	4.27	3.51			No epinephrin.
	7	18.69	5.52	3.38	0.0	2.66	Epinephrin resumed; deglycogenation complete.
	8	12.63	3.40	3.71			
	9	14.06	4.27	3.29	0.0		Fischer's solution 31 cc. by vein.
	10	16.88	4.43	3.81	2.31*	3.53	Glyceric aldehyde 5 gm. (5 per cent solution), also 31 cc. Fischer's solution by vein.
	11	13.31	4.47	2.98			Fischer's solution 31 cc. by vein. Note absence of retentions.
	12	17.94	5.66	3.17			Fischer's solution 31 cc. by vein. Blood in urine. Good recovery.

* Extra sugar reckoned on basis that G : N = 3.29, the ratio found in Period 9 with alkaline diuretic.

other six experiments and from the experiments performed with glyceric aldehyde in normal animals.

The diuretic alkaline solution selected consisted of 0.3 per cent Na_2CO_3 in 1.4 per cent NaCl solution, described by Fischer[14] for the clinical treatment of anurias, oligurias, edemas, etc. Other solutions could have been selected and might prove preferable for carrying out the principles of mild alkalinization and diuresis. But the Na_2CO_3—NaCl solution was chosen as containing no organic substance which could possibly be regarded as gluco-neogenetic and because we had previously used it successfully for similar purposes. It was injected into the vein slowly in doses of 20 to 30 cc. once in the 6 hour period. In all experiments one or more of the fore-periods were utilized as controls. In Experiment V, Period 10, the dog received 20 cc. of Fischer's solution by vein with no appreciable effect. There was indeed in this period some *decrease* of glucose below the figures of the preceding period; but this was to have been expected even if no therapy had been given, since in the preceding period epinephrin had been administered and, the animal having evidently retained a trace of glycogen, the urine for this period contained extra sugar. In Experiment VI, Period 10, Fischer's solution caused an increased output of nitrogen, but did not affect the glucose. It was given again in Period 12 with similar results. In Experiment VII, Period 9, it was given again and here caused a marked increase in nitrogen with a simultaneous rise in glucose, but nothing which could be mistaken for extra sugar since G: N was somewhat decreased. It was evident from these controls that the intravenous administration of Fischer's solution could not in itself cause any extra sugar to appear in the urine.

Having controlled in each experiment the effects of the alkaline diuretic solution alone, a similar dose was given after the administration of the glyceric aldehyde in each experimental period proper, with the results noted. In Experiment VII the solution was given in the fore-period, the experimental period, and in the first and second after-periods. The results were that the nitrogen showed a progressive increase to the end of the experiment with no such decrease in the glucose as char-

[14] Fischer, M. H., Oedema and Nephritis, New York, 2nd edition, 1915. 549.

acterized the after-periods of all other experiments. The urine of the last period, 17, contained blood which further abnormally increased the nitrogen. The animal recovered rapidly.

DISCUSSION.

Our chief interest in glyceric aldehyde lay in the fact that it might be regarded as glucose, split or dissociated but not otherwise altered, and that it might afford an available substance with which to test the theory that the diabetic defect consists in a decreased power of the tissues to dissociate glucose and perhaps allied substances. The remarkable ease with which glyceric aldehyde oxidizes and polymerizes outside the body in the presence of even small traces of alkali suggests that it is naturally more highly dissociated and might be more readily attacked in the diabetic body than glucose, as Neuberg stated to be the case, but did not prove. If it were more readily utilized in the body, the fact would have theoretical and clinical significance. The experiments show that although pure glyceric aldehyde is not wholly non-toxic, nevertheless it is borne without apparent injury by normal animals in single doses which would correspond to 150 or 200 gm. by mouth for a man of average size, so that its use as a food would not be impossible. When given to the diabetic it is capable, however, of becoming wholly transformed into glucose, and this transformation would appear to preclude the possibility of its having a greater utilization rate in the diabetic organism than glucose itself. It falls in this respect in the same category with glycerol, lactic acid, levulose, and several other substances which in the past have for unsound reasons been recommended in the treatment of diabetes.

The single fact that glyceric aldehyde may be converted quantitatively into glucose when it is introduced under the conditions of these experiments into the living body in a state of total diabetes would not finally settle the point of theoretical interest as to whether it might not be utilized in isolated diabetic tissues under other conditions, but certainly a mere failure to find in the urine of a diabetic individual the glucose equivalent of the glyceric aldehyde given does not justify the conclusion that the missing part has been utilized.

SUMMARY.

Chemically pure dl-glyceric aldehyde may be ingested by normal animals (rabbits and guinea pigs) in single doses of 1 to 2 gm. per kilo of body weight without noticeable symptoms, except diminution of urine. The same applies to subcutaneous doses of 0.3 to 1 gm. per kilo. A dose of 5 gm. per kilo by stomach was toxic but not fatal in a rabbit (diarrhea with unchanged glyceric aldehyde in passages, oliguria, albuminuria). A dose of 6.8 gm. by stomach killed a guinea pig. By the subcutaneous route, guinea pigs were killed with doses of 2.2 to 2.4 gm. per kilo, death being preceded by anuria. Autopsies showed slight passive congestion and parenchymatous changes in the abdominal viscera, especially the kidneys. No glyceric aldehyde was detected in the urine when urine was secreted. In completely phlorhizinized dogs and in human diabetes glyceric aldehyde may be converted into glucose. A virtually complete conversion is demonstrated if suitable precautions are taken to avoid toxic retentions due to the effects of glyceric aldehyde on the kidneys and tissues generally (small doses of glyceric aldehyde, alkaline diuretic therapy).

Concerning the technique of metabolism experiments with phlorhizinized dogs, it has been noted (a) that three hourly subcutaneous doses of 0.04 mg. of epinephrin per kilo of body weight generally serve to deglycogenize the animal within 24 hours without shortening the experiments or introducing undesirable effects; and (b) that by treating phlorhizinized dogs with a suitable alkaline diuretic solution to combat the diabetic acidosis and the added toxic effects of the experimental substance, the passage of metabolites into the urine may be facilitated.

STUDIES ON THE THEORY OF DIABETES.

VII. THE INTRAVENOUS TOLERANCE LIMIT FOR dl-GLYCERIC ALDEHYDE AND THE IMPROBABILITY THAT IT IS A CHIEF INTERMEDIATE IN GLUCOSE CATABOLISM.

By W. D. SANSUM and R. T. WOODYATT.

(*From the Otho S. A. Sprague Memorial Institute Laboratory for Clinical Research, Rush Medical College, Chicago.*)

We[1] have made a preliminary report concerning a quantitative pump devised for the purpose of making intravenous injections at exact rates for long periods of time, and of experiments in which different sugars have been injected at exact rates in different normal species and in certain pathological conditions in man. A detailed report is in the course of preparation. Certain results are of interest in connection with the preceding report on glyceric aldehyde.

By timed intravenous injections it has been found that pure d-glucose may be introduced into the peripheral venous blood of normal resting dogs, rabbits, and men at uniform rates below 0.8 gm. per kilo of body weight hourly for from 2 to 12 hours and presumably longer, without causing glycosuria. But when the rate of injection is increased to between 0.8 and 0.9 gm. of glucose per kilo per hour, glycosuria begins. The tolerance limit for glucose given by peripheral vein appears to be very close to 0.85 gm. per kilo of body weight per hour. This applies to glucose given in gram molecular (or 18 per cent) solution, and also to less concentrated solutions, provided that there is sufficient salt to maintain a suitable tonicity.

Pure crystalline dl-glyceric aldehyde, the same preparation as that used in the experiments described in the preceding article, has been injected into the ear veins of rabbits in 9 per cent aqueous

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[1] Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxv, 2067.

solution and in 2 per cent solution in 1.4 per cent salt solution, at exact rates, and it has been found that unchanged glyceric aldehyde begins to appear in the urine when the rate of injection exceeds 0.1 gm. of glyceric aldehyde per kilo of body weight hourly. The normal tolerance limit for dl-glyceric aldehyde when injected into the peripheral venous blood of rabbits lies close to 0.15 gm. per kilo per hour. The dl-glyceric aldehyde in the urine was detected by the power of the urine to reduce Fehling's solutions visibly within 1 minute at room temperature, and very markedly in 3 to 5 minutes, by its power to produce the characteristic slowly developing violet color with Schiff's decolorized fuchsine test for aldehydes, and by the absence of any optical variation in the urine which gave a zero reading in the polariscope before and after the appearance of reducing substance in it. It is clear from these experiments that the tolerance limit for intravenously administered dl-glyceric aldehyde (0.15 gm. per kilo per hour) is about ⅙ of that for glucose (0.85 gm. per kilo per hour). This does not imply, of course, that the body cannot utilize more than 0.15 gm. of glyceric aldehyde per kilo per hour if the rate of the injection into the peripheral vein is accelerated, but apparently it cannot use more without an overflow of glyceric aldehyde into the urine unless the urine is suppressed.

We have given d-glucose by peripheral vein to dogs for periods of 8 hours at the rate of 5.4 gm. per kilo per hour, without seriously impairing health, and at this rate of injection after the first 4 hours close to 2.7 gm. per kilo, or 50 per cent of the glucose given may be steadily excreted hourly, leaving 2.7 gm. per kilo hourly which continuously and permanently disappears. Most or all of this 2.7 gm. per hour must be utilized.[2]

If all the 2.7 gm. of glucose utilized were first split into glyceric aldehyde, then in these experiments we would in effect be introducing into the body 2.7 gm. of glyceric aldehyde per kilo per hour. But by experiment it has been found that this would be

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[2] If this were not the case, we should expect the physiological effects of accumulations of unburned glucose in the tissues; *viz.*, fall in the urinary output, thirst, fever, chills, and convulsions, which are seen when the rate of injection of glucose exceeds a certain value; or, avoiding dehydration by giving larger volumes of water, heart failure, and water logging. These effects were absent.

lethal, whereas the dogs which received 5.4 gm. of glucose per kilo per hour for 8 hours showed no serious effects. Also an injection rate of only 0.15 gm. of glyceric aldehyde per kilo per hour produces a definite triosuria, so that if even 5.5 per cent of the 2.7 gm. of glucose were converted into glyceric aldehyde we should expect to find easily demonstrable quantities of glyceric aldehyde in the urine, which we do not find. Even when giving 7.2 gm. of glucose per kilo per hour we have not succeeded in demonstrating glyceric aldehyde in the urine. Therefore it would seem impossible that more than 5 per cent at most of all the glucose which is utilized in the normal body is split into glyceric aldehyde.

The question arises as to what percentage of the 2.7 gm of glucose—unaccounted for in such an experiment—is actually oxidized, and how much polymerized or even reduced. Through the courtesy of Dr. Harvey Cushing, respiration experiments bearing on this question have been made possible. They were carried out by Dr. Walter M. Boothby and Miss Irene Sandiford of the Respiration Laboratory in the Peter Bent Brigham Hospital, Boston, the writers cooperating. The full report of this work will form a separate publication, but with the consent of the other laboratory, we may state that in one experiment a dog weighing 19.6 kilos received glucose steadily by vein for 13 hours at the rate of 3.522 gm. per kilo per hour and that after an equilibrium was established, the urinary excretion rate averaged 1.141 gm. per kilo an hour, leaving 2.381 gm. per kilo which disappeared hourly. The respiratory data obtained by Boothby and Sandiford would indicate that, roughly, 25 per cent of this 2.381 gm. was oxidized. In this case then the rate of glucose oxidation was approximately 0.6 gm. per kilo per hour. If only 25 per cent of this passed through a glyceric aldehyde phase, it would suffice to cause a triosuria. This rate of glucose oxidation can doubtless be greatly exceeded by employing higher injection rates and exercise, but the data given suffice to show that even in the oxidative utilization of glucose as distinguished from other chemical fates the change of glucose into two molecules of glyceric aldehyde can scarcely constitute a major step.

So far as we know, there are no experimental data which prove

that any glyceric aldehyde is formed in the breakdown of glucose in the body. But if there is, it represents a minor fraction. According to the theory under discussion in these papers, glucose exists in the body as saturated molecules in a state of dynamic chemical equilibrium with the ions or unsaturated residues into which it is dissociated. Among the dissociation fragments which participate in the glucose equilibrium, there could be some which might be designated as ions of glyceric aldehyde; *e.g.*, $CH_2OH - CHO - C\overline{OH} + H^+$, or as methylene derivatives of glyceric aldehyde, such as $CH_2OH - \underset{\wedge}{C} - COH + HOH$ or their equivalents. If glyceric aldehyde were introduced into the body it would by dissociating yield residues identical with these, so that glyceric aldehyde would be capable of entering into the glucose equilibrium, as noted in the preceding article. But it would not follow that any measurable concentration of molecular glyceric aldehyde should necessarily exist as such in the living body.

Many writers have regarded glyceric aldehyde as an important intermediate in the breakdown of glucose in the body. In the scheme of glucose catabolism constructed by Embden, and much quoted by others, the main path in the catabolism of glucose is depicted as an aldol depolymerization into optically active glyceric aldehyde which then changes into lactic acid. We believe that this particular change of glucose into glyceric aldehyde does not constitute an important step in its disintegration in the living body and that conceptions of the breakdown of glucose in which the process is depicted as though it proceeded from one fixed molecule to another are fundamentally inadequate.

EXPERIMENTAL STUDIES ON GROWTH.

I. METHODS.

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The series of articles of which this is the first will describe the results of experimental researches on the influence of certain dietary factors upon the process of growth and phenomena incident thereto in white mice. To accomplish the purpose of these researches it is imperative to exclude disturbances of growth due to factors, such as infections or exposure, other than those which the researches are designed to investigate. Accordingly before undertaking the experiments which will be described in subsequent communications of this series we devoted considerable attention and some preliminary experimental work to the construction of an environment for our experimental animals which would safeguard them as far as possible from zymotic diseases and enable us readily to prevent the spread of such diseases among them by infection from the sporadic cases which are practically impossible to avoid. It was also essential that the environment should be such as to exclude, as far as could be foreseen, all other variable factors which might conceivably exert an appreciable effect upon the growth of the animals. The methods which we have contrived to meet these desiderata have proved highly satisfactory, inasmuch as we have been able to maintain over 75 per cent of our control animals in healthy condition to the age, at the date of writing, of nearly 18 months, the great majority of deaths among adult animals having been due either to injuries attributable to the belligerent habits of the males or to the spontaneous incidence of tumors. Moreover, the methods are very convenient, permitting rapid manipulation, so that, when necessary, one individual with little assistance can maintain entire charge of about 500 animals. Since a descrip-

tion of the methods employed must in any case precede the description of the experimental results obtained by their aid we have thought it advisable to devote the first article of this series to this purpose.

The essential factors in the environment of an animal in captivity may be summarized under the following heads: (1) the construction of cages and nests; (2) the cleansing of the cages and nests and the removal of debris; (3) the methods of administering food and water; (4) the character of the food administered; (5) the condition of temperature, humidity, etc.; (6) the methods of combating the spread of infections; while in experiments upon growth involving the weekly or biweekly weighing of 400 to 500 animals, the methods employed in identifying and weighing the animals are also of considerable practical importance. These various factors will be dealt with *seriatim.*

Construction of the Cages and Nests.

The cages which we employ are constructed, with some modifications essential to our special purpose, from the design recommended by Daniel.[1] In this we have been greatly assisted by the advice of Dr. Daniel, to whom we desire to express our indebtedness.

Our cages differ from those illustrated by Daniel in the article to which reference has been made, in the following particulars. Since we did not administer milk to our animals the milk receptacle is eliminated. Since we employ a different device for administering grain the food funnel and food cup illustrated by Daniel are eliminated. The nest boxes occupying only about two-thirds of the width of each compartment, a narrow runway is left between the side of the nest and the partition of the adjacent compartment (or side of the cage) on the right, the water receptacle extending down into this runway so as to be conveniently reached by a mouse from the wire mesh floor of the compartment. This modification was introduced because it was found that if the opening of the water receptacle was directly over the nest box as in Daniel's design, water tended to collect on the roof and seep into the interior of the nest box; furthermore,

[1] Daniel, J. F., *Am. Naturalist,* 1912, xlvi, 591.

the surfaces of the nest boxes which we employ being slippery, if they had extended from side to side of the compartment the mice could not have climbed them to reach the water. Incidentally our arrangement afforded the mice more running-space and, since they utilized the water receptacle as a means of climbing up and down to and from the roof of their nest boxes, it afforded them a healthful degree of exercise. Finally, the back of the cage, as well as the front, sides, and partitions, consisted of window-glass of medium thickness fitting into grooves in the wooden frame, the galvanized iron back employed by Daniel being deemed undesirable on account of the possible chemical action of stale urine upon it, leading to the formation of metallic salts which might be absorbed by the animals and exert an unknown effect upon their growth and well-being. The danger of this in the case of the wire mesh floor was much less because moisture would tend to collect on the lower side of the mesh, remote from the animals, and incrustations would tend to be displaced by the movements of the animals and fall down to be collected and removed along with the other debris.

Fig. 1 shows our cage from the front, with nests (A), food tubes (B), water tubes (C), and receptacles (E and F), *in situ*. The glass sheets forming the sides and partitions of the cage are fitted into grooves cut in the upper part of the wooden frame, while the sheets of glass forming the front and back are held in place below the upper part of the frame by the sides and partitions which accurately fit the space between them. The top of the cage is formed, at the back, of a strip of wood $2\frac{1}{2}$ inches broad, perforated with holes for the water receptacles to pass through and provided with rests for the hinged lid $11\frac{1}{2}$ inches wide which is attached to it and forms the front part of the top of the cage. The whole of the top part of the cage is removable, being attached to the body of the cage by three hooks. The floor and the front or hinged portion of the top of the cage are covered with $\frac{1}{4}$ inch wire mesh, and the glass sides, front, back, and partitions are so arranged that at no part of the cage can the animals gain access to any wooden surface excepting when, as occasionally happens, they climb upside down on the lid of the cage. This arrangement prevents the wetting of wood by urine and the unhygienic conditions arising therefrom.
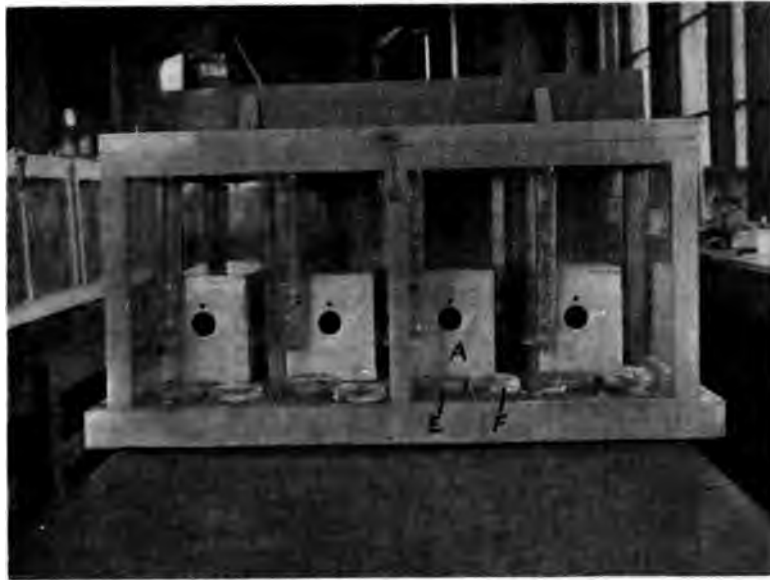
Fig. 1. Mouse cage with nests (A), food tubes (B), water tubes (C), waste grain tray (E), egg receptacles (F), and grain tube stoppers (D), *in situ.*
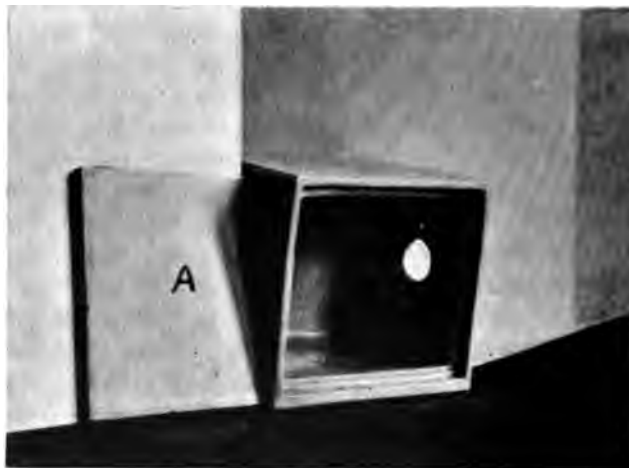
Fig. 2. Terra-cotta nest box. (A) removable side.

The dimensions of the cages are approximately as follows: length of top of cage 27½ inches, width 13¾ inches, width from front to hinge 11¼ inches; length of base of cage 29¼ inches, width 15 inches; total inside length of cage 25¾ inches, width 11½ inches.

The nests which we employ[2] are made of terra-cotta glazed on every surface which is accessible to the animals. Fig. 2 shows the general construction. They are 5¾ inches high, 6¼ inches from back to front, and 4¾ inches wide, all outside measurements, the walls being $\frac{5}{16}$ of an inch thick. The inside surface of the bottom of the nest is channeled and slopes towards the middle, where a small orifice discharges moisture. The entrance to the nest is circular, measuring 1¼ inches in diameter. The lid or loose wall A fits into the side of the nest where it rests against a rim which projects about ¼ inch from the inner walls of the nest on all four sides; the inner surface of this lid is glazed while the outer, which is pressed up against the left hand partition or side of the cage, is unglazed. This arrangement is preferable to having the loose wall of the nest forming the roof, as this would allow urine to seep in around the edges. Moreover, this arrangement permits one surface of the loose wall, namely, that which is inaccessible to the animals, to be unglazed, which fact facilitates the manufacture of the cages. The only other unglazed surface is the under surface of the base of the nest, a surface which is also, of course, inaccessible to the animals. We prefer terra-cotta nests to wooden or metal nests for a variety of reasons. Wooden nests tend to become sodden with urine and any waterproofing substance would be gnawed by the animals and might affect them deleteriously; moreover, wooden nests are not easily sterilized. Metal nests are subject to more sudden fluctuations of temperature and, moreover, we desired to avoid metal surfaces for reasons outlined above. The terra-cotta nests retain for a considerable time the warmth which the animals communicate to them, thus securing equable conditions of temperature within the nests, and, whenever it appears desirable, the entire nest can be plunged into mercuric chloride solution and then, after careful washing and drying, be employed again without fear of communicating infection by its use.

The nests are manufactured for us by the Steiger Terra-Cotta Co. San Francisco, at a cost of 60 cents each.

The small perforation seen in the photograph above the entrance to the nest was meant for a purpose which was abandoned early in the experiment and is unessential to the design of the nest.

The bedding employed was a vegetable fiber known to the local trade as "silk floss." It is used for upholstering and is probably identical with or similar to the fiber known elsewhere as "kapok," which is the floss or seed-hair of *Eriodendron anfractuosum*. This floss has the advantage of not matting so readily as absorbent cotton, and, moreover, it does not cling to the animals as absorbent cotton tends to do. Only in certain well defined pathological conditions was any tendency noticed for the floss to adhere to any part of the animal. The supply of floss in any one nest should not be too abundant, otherwise the animals take the initiative themselves and eject the excess from the nest. In hot weather they frequently draw the bedding out of the nests and sleep outside, replacing the bedding and retiring within the nests of their own accord as the temperature falls.

Removal of Debris and Cleansing of Cages.

The cage shown on the left hand side in Fig. 1 gives an idea of the arrangement of the cages. They were placed end to end and back to back in rows five cages long and two deep, on tables of which the edges are raised about $1\frac{1}{4}$ inches, thus forming a sort of tray. Along the entire length of the top of the table was laid a sheet of stout wrapping paper, and the ends of the cages were supported by narrow wooden slats laid across from edge to edge of the table at the requisite intervals. The debris from the cages thus fell through the wire mesh floor on to the wrapping paper. Once a week the cages were lifted down, and the debris was shaken into the middle of the paper which was then folded up and removed, following which a fresh sheet of paper was laid down and the cages were replaced. The removal of debris by this means was expeditious and thorough and this plan was preferred to the zinc lined tray flushed by water which is recommended by Daniel, first because it was found that the removal of debris by this means was not so rapid as that just described, and secondly because we feared the effect upon the well-being of the animals of the frequent presence of water underneath the cages.

The cages were cleansed once a week. This was readily accomplished by removing the top of the nest, washing the glass surfaces with a wet cloth, and subsequently drying them, and scrubbing the wire screen floor with a dry brush provided with stiff wire bristles. From time to time the cages were more thoroughly cleansed by removal of the glass sides, ends, and partitions, and thoroughly scrubbing the woodwork and wire screen with soap and water. The nest boxes and bedding were renewed once a week, and the used nests were thoroughly washed and dried before reemployment.

Nature of the Food and Method of Administering Food and Water.

The object of the experiments, except in certain instances to be specified later on, being to study the effect of abnormal additions to an abundant, normal, and well mixed diet, a wide variety of food was given to the animals and a constant supply of food was offered to them. The staple consisted of rolled barley which was supplied to them by means of the receptacle marked B in Fig. 1. This consisted of a heavy walled glass test-tube 9 inches long and $1\frac{1}{4}$ inches in diameter, a hole being blown in the lower end large enough to readily permit the egress of a single grain. Thus contamination of the grain by feces is avoided with the possible spread of infection involved therein, and, moreover, the animals are put to some trouble to get the grain and are not so wasteful of it as they are when it is supplied to them in open vessels. Even so, however, they frequently amused themselves by pulling out grains and throwing them down, with the result that they fell in large quantities through the wire screen uneaten. In order to minimize this waste the shallow glass trays E (Petri dishes) were introduced to catch this grain and keep it accessible to the mice for a brief period until consumed. These trays were kept in position under the opening of the grain tubes by means of a narrow strip of aluminum foil bent into a double hook, one arm of which was bent over the edge of the tray and the other under the adjacent partition. The tubes were suspended by a wire hook from the top of the partition. At first they were left open at the upper end, but it was found that the animals not infrequently climbed up to the top of the tube and attempted to reach the

grain from above, with the result that two or three fell head first into the tubes and were suffocated before they were discovered. Hence the grain tubes were closed at the top by the removable stopper D which consisted simply of a short length of glass tubing of about ⅓ inch in diameter and 1 mm. in thickness of the wall, one end being sealed and then blown out into a bulb and the tube being then cut in such a way as to leave the bulb provided with a short neck which was inserted into the grain tube.

The grain tubes were replenished twice daily with the aid of a scoop and a metal funnel fitting into the top of the tube.

Another item of the dietary which is supplied daily (except Sundays) is the mixed white and yolk of eggs. The whites and yolks of eggs are beaten up together and strained, 5 cc. of the mixture being supplied to six mice (i.e., to each compartment). The mice eat this greedily and it is usually completely consumed a few minutes after being offered to them. This was made the vehicle for administering the special substances the action of which upon growth we wished to determine. The egg was supplied in Syracuse watch glasses (F in Fig. 1) which were washed and renewed daily.

Fresh leaves of lettuce were supplied twice weekly, and on Sundays thoroughly dried bread. It is essential that bread supplied to mice should be thoroughly dry, otherwise intestinal disturbances make their appearance. Our procedure is to cut the bread into small cubes and dry thoroughly over a radiator. Only ordinary bakers' bread was employed, French and other fancy varieties being avoided.

The water is administered as in Daniel's cages in glass tubes sealed at the upper end, with the lower end melted down until only a small orifice remains. Instead, however, of holding them in place by rubber bands we found it more convenient to accomplish this by blowing a bulb at the sealed end, too large to pass through the orifice in the cover of the cage. The tubes employed are ½ inch in diameter and 11½ inches long and are made out of glass tubing of about 1 mm. in thickness of the wall. They are filled daily or every second day. From time to time they are cleaned out with the aid of a fine brush of the type generally listed in catalogues as "glass tube cleaners," cut in half and soldered on to a length of stiff steel wire. For filling the drinking

tubes we employ a large wash bottle provided with a rubber bulb (without a valve) attached to the air inlet tube of the bottle by a convenient length of rubber tubing. In this way pressure could be communicated by pressing the bulb, and it could be instantly reduced, if desired, by simply relaxing the bulb. The water exit tube is attached by means of a short piece of rubber tubing to a short length of tubing drawn out into a point which fits conveniently into the orifice of the drinking tubes.

The Conditions of Temperature, Humidity, etc.

The room in which the animals were kept was large and well ventilated and lighted, being supplied with a row of windows on two sides.

The source of heat was a single steam heated radiator provided with a valve permitting the occasional escape of small puffs of cooled steam. This, together with the constant ingress of fresh air (but not wind) kept the atmosphere humid but not unpleasantly so. At a late hour in the evening (about 10 p.m.) the steam pressure began to fall owing to the banking of the fires. Thereafter the room cooled down slowly until an early hour in the morning when the fires were renewed and the steam pressure rose again. On warm days the steam heat was turned off altogether, while on the other hand a small electric radiator was employed in exceptionally cold weather. The source of heat was in a corner of the room remote from the cages, so that the temperature conditions over the area occupied by the cages were tolerably uniform.

During 5 months, fairly representative of the entire period occupied by the experiments, the temperature of the room was registered by a self-recording thermometer. The mean temperature at midday during this period was 20.9°C. The mean diurnal maximum (from 4 to 6 p.m.) was 24.1°C., and the mean diurnal minimum (from 6 to 8 a.m.) was 17.5°C. The mean variation in temperature throughout the 24 hours was 6.6°C. The highest temperature recorded was 31.1°C., and the lowest, on one occasion only, owing to a failure of the steam heat, was 9.4°C.; with this exception the lowest temperature recorded was about 12°C.

The temperature was therefore subject to considerable fluctua-
tion, a condition which, according to Chalmers Mitchell,[3] is con-
ducive to the well-being of animals in captivity.   Extreme vari-
ations, such as those found by Daniel to be deleterious,.did not,
of course, occur, the maximum variation noted in any 24 hours
being 12°C.

Methods of Combating Infection.

White mice are fairly hardy animals, the only disease to which
they are especially subject being β-paratyphoid.[4]   This made
its appearance in a certain number of animals about 4 or 5 weeks
after mixing different litters;[5] i.e., when the animals were from 8
to 9 weeks old.   From 10 to 15 per cent of the animals then dis-
played symptoms of diarrhea and emaciation, passing blood-
stained feces.   These animals were promptly killed, the records
of their weights discarded, and they were replaced by fresh ani-
mals, the infected cages, food and water receptacles, etc., being
washed with alcohol.   This stage past, hardly any further cases
of paratyphoid were observed, and, save in one instance when a
whole compartment (six) of adult animals suddenly developed
symptoms, the cases observed were confined to a single mouse in
a cage and led to no epidemic.   The glass partitions naturally
prevented the spread of infection from one compartment to the
adjacent compartments.

Among adult animals sporadic cases of pneumonia occasionally
occurred.   Only in one instance when three animals in one com-
partment had developed the disease before it was detected did
anything in the nature of an epidemic occur.   The symptoms
were emaciation and severe dyspnea.   The animals were killed,
or, in doubtful cases, isolated for further observation, and the
infected nests and cages sterilized in the manner described above.
Postmortem examination in these cases showed the lungs to be
engorged and blood-red.   Cancer of the lungs which occurs fre-
quently among female mice resembled pneumonia in external

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[3] Mitchell, P. C., Proc. Zool. Soc., London, 1911, 469.

[4] Infected animals were examined for us by Dr. K. F. Meyer, who identi-
fied the infection as β-paratyphoid.

[5] It was not so frequently observed when the litters were kept segre-
gated.

symptoms, but the postmortem appearance of the lungs, of course, differed too greatly from that of pneumonia to permit any uncertainty as to the cause of death.

Infected wounds, due to injuries received in fighting, were observed occasionally among the males. If the infection was serious the animal was killed to avoid confusion of the records by the inclusion of possibly abnormal figures.

The animals were frequently examined for external parasites, which according to both Daniel and Chalmers Mitchell are deleterious to the well-being of animals in captivity. None were ever found. In one or two instances animals which came to postmortem had a small cyst on the surface of the liver containing a long worm coiled up in it. In each instance death could be attributed to another cause and the animals appeared to have been in no appreciable degree affected in their health by the presence of the parasite. With this small number of exceptions internal parasites were not observed in any of the animals which came to autopsy.

The weights of animals found suffering from infections, cancer, or dying from unknown causes, were deleted from the records for a period of 5 weeks preceding death. This was deemed ample allowance to exclude abnormalities in weight attributable to the diseased condition, for pneumonia develops rapidly, death occurring within 2 or 3 days after the first symptoms are observed. Paratyphoid produces very obvious emaciation and feebleness within a like period, and cancers which could be observed externally usually reached the stage of ulceration within 3 weeks after the first small lump was detected.

Methods of Identifying and Weighing the Animals.

The animals were identified by means of nicks and holes punched in the ears, a small punch such as is commonly employed by chicken breeders for marking their birds being used for this purpose. The different classes of experimental animals, *i.e.*, controls, pituitary-fed animals, etc., were distinguished by means of punch-holes in the ears, to guard against any possibility of accident to the cages leading to temporary mixing of the animals. This contingency never arose and the punch-holes were therefore never

required for purposes of identification.   The animals, six in
number and of the same sex, in each compartment were differ-
entiated by means of nicks in the ears and distinguished in the
records by numbers and the letter designating the compartment,
the capital letter designating the experimental class, and the
symbol designating sex.   The woodwork of the cages contain-
ing different experimental classes was painted in different colors
as a mnemonic guide.

The sexes were kept separate to avoid complication of the data
by pregnancies.

The time consumed in the weekly or biweekly weighing of so
many animals was a serious consideration.   It was necessary to
weigh to the desired degree of accuracy as rapidly as possible.
Ordinary balances were too sensitive and their period of oscillation
too long.   Spring balances were tried and found insufficiently
accurate.   Finally we employed and found in the highest degree
satisfactory a modified form of the triple beam balance.[6]   The
balance, as modified, is shown in Fig. 3.   The modifications which
we added consist of the brass cup C into which dips a disc-shaped
plunger affixed by a length of stiff wire to the under side of the
lower beam.   The wire was cut to such a length that the disc
could not touch the bottom of the cup nor emerge from the sur-
face of the liquid paraffin ("medicinal petroleum") with which
the cup was filled.   This damped the oscillations of the balance,
causing it to come almost instantly to equilibrium.

The threaded milled brass counterpoise B and low pitched
screw-thread A which permit its accurate adjustment were at-
tached to the front of the beam by means of brass posts screwed
into the lower ends of the beam.   This counterpoised the beaker
in which the animals were placed to be weighed.   The capacity
of the balance, after counterpoising the beaker, is 111 gm., it
reads to 0.1 gm., and is sensitive to 1 or 2 cg.   Animals of 4 weeks
of age or over were weighed to the nearest 0.5 gm., the daily
variation equalling or exceeding this amount.   Younger animals
were weighed to the nearest 0.1 gm.

With the aid of this balance, which with suitable modifications
of counterpoise and capacity could be readily adapted for weigh-

[6] This balance is illustrated under No. 330 in Catalogue C issued by
Eimer and Amend, New York, 1913.

ing other small animals, the time consumed in weighings is reduced to a minimum, it being possible without undue haste, with clerical assistance in taking down the observed weights, to weigh no fewer than sixty animals in an hour, the time consumed in removing the animals from their compartments, renewing the nest box,
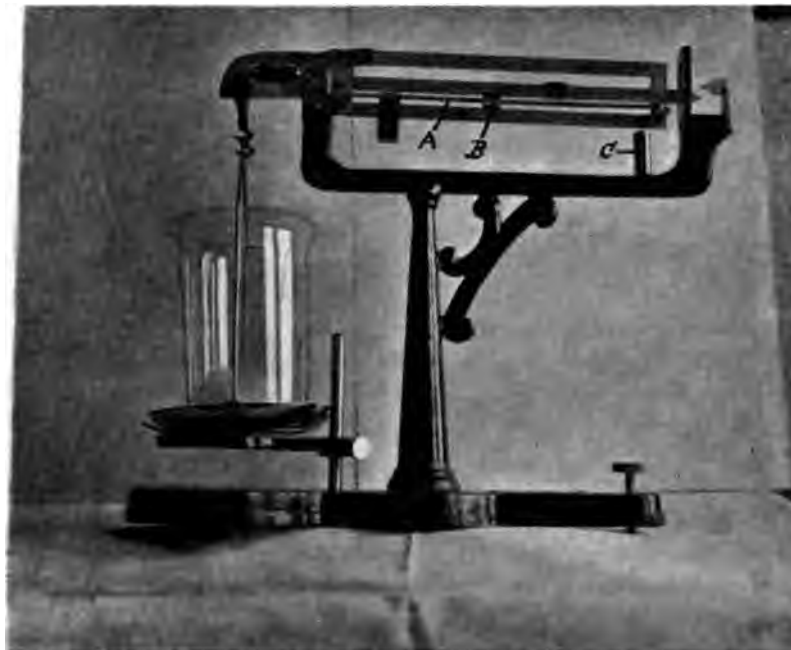
FIG. 3. Balance employed in weighing mice, showing screw-thread (A), and milled brass counterpoise (B). A plunger attached to the lower end of the beam dips into liquid paraffin contained in the brass cup (C).

and replacing the animals when weighed being included in this estimate.

We have weighed mice at short intervals throughout the day from 8 a.m. to 10 p.m., and in this way have found that their weight is subject to fluctuations occurring at fairly definite times in the day, dependent upon the feeding habits of the animals. A favorite time for feeding would appear to be the early evening, the weight always being high at about 10 p.m. In the early

morning the weight is again low and rises during the morning to fall again during the afternoon. We therefore always weighed the animals at the same time; namely, the middle of the afternoon, when the amount of material contained in the alimentary canal is relatively small.

Sources of Error in the Weighings.

In order to ascertain the maximal possible fluctuation in weight attributable to the contents of the alimentary canal and bladder, we permitted six 4 months old mice to fast for 27 hours, depriving them also of water during the last 4 hours of this period. The curve of weight of mice deprived of food shows a sharp break at 6 hours, indicating that at the end of this period the intestines are completely emptied. Hence the alimentary canals of these mice were certainly empty of food at the end of the period indicated and no small proportion of their loss of weight was due to actual inanition. We now weighed the animals, then supplied them with an abundant meal of every variety of food, and replaced their drinking tubes. At the end of 1 hour, when they had ceased eating with voracity, the average gain in weight was 5 per cent of the initial (non-fasting) weight. This, therefore, may be regarded as the extreme possible fluctuation in weight due to varying intestinal and bladder contents. The fluctuations actually encountered must, of course, have been considerably less than this, probably not exceeding 1 or 2 per cent of the body weight.

The animals, as stated, were weighed to the nearest 0.5 gm. subsequently to 4 weeks of age (12 gm. weight in males, 10.5 in females), being weighed to the nearest 0.1 gm. previously to this. From this source, therefore, an error of 0.25 gm. may possibly arise in the recorded weight of an individual mouse. This would lead to an error of 2 per cent in the recorded weight of a mouse of 4 or 5 weeks of age, and less than 1 per cent in the recorded weight of an adult mouse. The error thus introduced into the mean weight of a number of mice would, of course, be much less than this, since in the long run plus and minus errors due to this source of inaccuracy would tend to cancel each other.

SUMMARY.

Methods employed in experiments on the growth of mice hereafter detailed are described by which the animals may be maintained in good health and as free as possible from zymotic disease and other deleterious factors in the environment which might conceivably exert an effect upon their growth.   Improved forms of cages, food receptacles, and nest houses are described.

Rapid methods of handling and weighing large numbers of animals, and a balance especially adapted to this purpose, are also described.

EXPERIMENTAL STUDIES ON GROWTH.

II. THE NORMAL GROWTH OF THE WHITE MOUSE.

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INTRODUCTION.

Nature of the Growth Process.

I have shown in previous communications[1] that the growth of man and of animals consists of a number of phases or growth cycles which succeed one another and to some extent merge into one another at the transitional period. These transitional periods are critical periods in the growth of the animal, and if for any reason accurate linkage with the succeeding cycle fails, the life of the animal or individual is imperilled.

Each of these cycles of growth is characterized by an initial period of slow growth succeeded by a period of rapid growth, and that in turn by a period of slow growth, the entire cycle forming a single S-shaped curve which is symmetrical about its center or moment of maximum velocity.

The chemical syntheses which constitute the growth of an animal are therefore of such a nature that during the first half of any given growth cycle the velocity of synthesis is progressively increased in proportion as it has already proceeded; in other words, it is autocatalyzed or self-accelerated and, like all autocatalyzed reactions, in the latter half of the cycle the syntheses are retarded by the progressive accumulation and mass action of the products of the synthesis. In the communications to which

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[1] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 581; 1908, xxvi, 108; 1913, xxxvii, 497; *Univ. California Publications, Physiology*, 1914-15, iv, 211; *Am. J. Physiol.*, 1915, xxxvii, 1, 74.

reference has been made, it has been shown that the formula of autocatalysis,

$$\text{Log}\frac{x}{A-x} = K\,(t-t_1)$$

(where x is the increment in weight attained in any one cycle at time t, A is the total increment in weight attained in the cycle, t_1 is the time at which the cycle is half completed, and K is a constant expressing the specific velocity of the process), accurately represents the quantitative relationships which have been observed in those instances where accurate measurements made upon a sufficient number of individuals have been available.

Catalysers of Growth.   Objects of the Experiments.

The fact that the syntheses which constitute the growth of an organism are autocatalyzed, implies the existence of substances which are capable of acting as catalysers of growth. Nor is direct evidence lacking to this effect.   We know that many of the glands of internal secretion, notably the anterior lobe of the pituitary body, the thyroid, and the thymus, are capable of exerting important influences upon the processes of growth,— influences the lack of which becomes apparent in ways which are now familiar when one of these glands is removed by operation or injured by disease.   These glands are universally supposed to exert their influence by means of secretions which are thrown into the blood stream and which must therefore contain positive or negative catalysers of certain phases or types of growth.   Moreover, direct evidence has been adduced by various investigators to show that certain substances, notably lecithin and cholesterol, exert an accelerating or retarding influence upon certain types of growth.[2]

In this connection it is a fact of great significance that both lecithin and cholesterol, while notably accelerating certain types

[1] King, H. D., *Biol. Bull.*, 1907, xiii, 40.   Johnson, M., *Univ. California Publications, Zoology*, 1913, xi, No. 4.   Bain, W., *Lancet*, 1913, clxxxii, 918.   Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvii, 497. Robertson, T. B., and Burnett, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1912-13, x, 140; *J. Exp. Med.*, 1913, xvii, 344.   Browder, A., *Univ. California Publications, Physiology*, 1915, v, 1.

of growth, exert a no less notable retardation upon certain other types, and, furthermore, that those types of growth which are accelerated by cholesterol are retarded by lecithin, and *vice versa*.

This fact leads directly to the conception outlined in a previous communication;[3] namely, that of the occurrence of two different types of growth which I have designated the autokinetic and the autostatic types, respectively, the former type being that in which the growth accelerator progressively increases in amount, the latter that in which it progressively diminishes in amount; either process is, as I have shown in the communication to which reference has been made, quantitatively expressible by the auto-catalytic formula. For reasons which are described in detail in the same article, increase in the concentration or available mass of the catalyser may be expected to lead to an acceleration of the latter half of an autostatic or the initial half of an auto-kinetic cycle, a period of growth which we may inclusively term the "anatachytous phase," while a like increase in the concentration or available mass of the catalyser may be expected to lead to the retardation of the latter half of an autokinetic or the initial half of an autostatic cycle, a period which we may in like manner inclusively term the "katatachytous phase" of growth ($\tau\alpha\chi\nu\tau\eta\varsigma$, velocity).

The possibility, or rather probability must not, of course, be lost sight of that many different catalysers of growth exist, each of which may not improbably be furnished to the organism from a different source (*e.g.*, gland of internal secretion). Nor is it at all unlikely that the growth of certain tissues, such as the sub-cutaneous or the bony tissues, may be controlled by special catalysers of their own. Furthermore, specific retarders of certain phases of growth may exist, acting either directly as negative catalysers, or indirectly by diminishing the available mass of or rendering inoperative a positive catalyser of growth. Nevertheless, broadly speaking, the action of all these substances may be expected to conform to the general characteristics outlined above.

The object of the present series of experiments is to elucidate if possible, by the direct administration of substances known or suspected to exert an influence upon growth, the chemical nature

[3] Robertson, *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvii, 497.

and mode of action of the various catalysers of growth. Owing to its hardiness, rapid growth, smallness, and fecundity, the white mouse was chosen as the experimental animal. But for this purpose it was necessary to establish an accurate norm or curve of growth of normal animals obtained under conditions precisely resembling those which prevailed among the animals receiving the known or suspected catalysers. Such a norm for the white mouse is not available, and in any case, if previously established, might not necessarily apply to the race and environment employed in this experiment. The present article is designed to present the data which furnish such a norm. In addition to the growth, other factors in the life history of the animals were studied, with a view to making the accumulated data as complete a résumé of the changes involved in the growth of these animals as it was feasible to obtain.

Methods of Breeding, Weighing, etc.

The breeding animals were kept in oil cans perforated at the bottom, with one side cut out and covered with wire gauze. Wooden nest boxes were employed. In all other respects, save that they were fed with finely chopped hard boiled egg instead of with raw egg, the animals were treated in exactly the same way as the experimental animals.[4] Pregnant animals were placed in cans by themselves as soon as their condition was discovered.

The young were weaned at 3 weeks after birth, as recommended by Daniel,[5] and from 1 to 2 weeks later, *i.e.*, at the age of 4 to 5 weeks, they were removed to the cages described in the preceding article and handled and fed as therein stated, the sexes being segregated.

The animals were weighed upon the day of birth and at 7 days, without any attempt at differentiation of the sexes. Thereafter, the animals were marked with spots of methylene blue.[6] The records of each animal were kept separate and the sex of

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[4] Compare the preceding article on Methods.

[5] Daniel, J. F., *Am. Naturalist*, 1912, xlvi, 591.

[6] At this age, when the hair begins to grow, it is easy to mark the animals in this way if the hair be first moistened with a little 95 per cent alcohol and then stained with a concentrated aqueous solution of methylene blue. These marks last several weeks.

each animal was determined when the sexual organs became sufficiently differentiated to render this an easy matter. Weighings were repeated on the 11th (9th to 13th inclusive), 14th (exact), 17th (15th to 19th inclusive), 23rd (22nd to 24th inclusive), and 28th (25th to 31st inclusive) days, and thereafter once a week until the age of 210 days, after which the weighings were biweekly. Animals weighed on the 25th to the 31st days, inclusive, after birth, were considered to have been weighed on the 28th day, those weighed on the 32nd to the 38th days inclusive to have been weighed on the 35th day, and so forth. Animals weighed on the 218th to the 231st days inclusive were considered to have been weighed on the 224th day, animals weighed on the 232nd to the 245th days inclusive to have been weighed on the 238th day, and so forth, the animals weighed being sufficiently numerous and sufficiently scattered over these gaps of time to permit this to be done without introducing any appreciable error. The animals (including and subsequent to the 4th week) were always weighed at the same time of day; namely, the middle of the afternoon.

Period of Gestation. Size of the Litter.

According to Daniel,[5] the period of gestation in the white mouse is 20 days.

From June 19, 1914, to July 25, 1915, inclusive, the total number of animals born in the laboratory was 1,242. The number of litters was 241. Thus the average number of animals born in one litter was 5.15 or, in round numbers, 5.

The standard deviation of the size of the litter was determined in the manner usual in statistical investigations;[7] namely, by squaring the deviation of each litter from the mean (5), adding these squared deviations, dividing the sum by the total number of litters, and taking the square root of the quotient. The standard deviation was in this way found to be 2.2. The percentage ratio of the standard deviation to the mean is the measure of the variability of the quantity measured. It expresses the percentage of deviation upon either side of the mean within which two-thirds of the observed measurements may be expected to

[7] Davenport, C. B., Statistical Methods, New York, 2nd edition, 1904, 15. Yule, G. U., An Introduction to the Theory of Statistics, London, 2nd edition, 1912, Chapter 8.

lie.[8] In the case of the size of the litter delivered by the white mouse the variability is extremely ·high, no less than 42.8 per cent, which means that two-thirds of any large number of litters may be expected to lie between 2.95 and 7.35. In round numbers over two-thirds of the litters may be expected to consist of 3, 4, 5, 6, or 7 young. The largest observed litter contained 11 young.

Of all the animals born in the laboratory during the period mentioned, 358, or 29 per cent, died from various causes (inanition, paratyphoid, being eaten òr otherwise injured by the mother) before they were weaned.

There was a noticeable tendency for some large ·litters to suffer from lack of nutrition, especially towards the end of the lactation period. Occasionally such litters were divided up among other nursing mothers, with good results. Animals that were noticeably abnormal or in poor condition were, of course, not utilized for weight measurements. In order to avoid any possible selection, the litters were taken by chance, one class of experimental animals (*i.e.*, normals, pituitary-fed, etc.) being usually completely filled (with the exception of those subsequently added to replace early deaths from paratyphoid) before another class was started. Hence the norm herein presented offers as nearly as possible a true picture of the normal growth of all the experimental animals.

Growth Curve of Normal White Mice.

Tables I and II show the average weights at the ages chosen of male and female mice respectively, the figures for the weights at birth and 7 days referring to mixed males and females. The variability of the weight at each age, determined in the manner described above, is included in the third and the number of animals weighed in the fourth columns of each table. These results are also depicted graphically in Figs. 1 and 2, the abscissæ being time and ordinates weight, the smooth curves representing the progressive alterations in weight and the irregular curves the alterations in variability (ordinates, percentage) with age. In the growth curves the dots represent the actually determined

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[8] More exactly, 68.27 per cent; compare Davenport, Statistical Methods, New York, 2nd edition, 1904, 16.

weights; in the variability curves the observed points are joined by straight lines.

The following characteristics may be especially noted in these curves: In each sex there are three separate extra-uterine growth

TABLE I.

Normal Male Mice.

Age.	Weight.	Variability.	No. weighed.	Age.	Weight.	Variability.	No. weighed.
	gm.	*per cent*		*wks.*	*gm.*	*per cent*	
At birth.	• 1.23	11.4	56	21	26.28	11.2	35
days				22	26.06	9.0	35
7	3.31	19.2	45	23	26.34	9.8	34
11	4.67	19.3	41	24	26.82	10.1	34
14	5.14	19.1	24	25	27.05	11.0	33
17	5.70	24.7	43	26	26.94	10.8	33
23	9.32	18.5	45	27	26.55	11.3	32
wks.				28	27.19	10.9	31
4	12.38	24.4	65	29	27.08	10.9	31
5	12.45	24.6	117	30	27.23	9.5	31
6	15.58	22.0	72	32	27.61	9.6	32
7	18.08	16.9	43	34	27.71	10.5	31
8	19.36	15.9	42	36	27.95	10.1	30
9	20.63	16.5	42	38	28.20	10.5	30
10	21.19	16.7	42	40	28.27	10.4	30
11	21.81	13.3	42	42	28.28	11.2	30
12	22.65	13.7	42	44	28.62	12.0	30
13	23.31	14.2	37	46	28.55	11.7	30
14	23.96	14.1	36	48	28.22	10.8	30
15	24.28	11.9	36	50	28.88	11.1	30
16	24.75	12.7	36	52	28.68	10.7	30
17	25.21	12.4	36	54	28.67	12.0	30
18	25.61	12.1	36	56	29.32	12.0	30
19	25.81	12.1	35	58	29.10	10.9	30
20	26.10	10.8	35	60	29.08	11.8	30

cycles, just as in the growth curve of human beings.[9] In the males the first cycle (I) attains its maximum velocity at some time between birth and 7 days, nearer the latter than the former, and culminates at 14 days. The second cycle (II) begins coincidently with the culmination of the first, attains its maximum velocity at 21 to 23 days, and culminates soon after the 28th day.

[9] Robertson, *Am. J. Physiol.*, 1915, xxxvii, 1.

Here a definite pause in growth occurs, but the third growth cycle
(III) begins to show its effects within the succeeding week, at-
tains its maximum velocity rather rapidly, at about 6 weeks, and
thereafter decreases continuously but slowly in velocity so that

TABLE II.

Normal Female Mice.

Age.	Weight.	Variability.	No. weighed.	Age.	Weight.	Variability.	No. weighed.
	gm.	*per cent*		*wks.*	*gm.*	*per cent*	
At birth.	1.23	11.4	56	21	23.06	12.3	36
days				22	23.32	12.5	36
7	3.31	19.2	45	23	23.51	12.0	35
11	4.58	23.0	33	24	23.68	11.3	36
14	4.91	22.5	17	25	23.79	11.4	35
17	5.48	25.3	35	26	24.04	11.6	36
23	8.45	18.2	37	27	24.00	11.8	35
wks.				28	23.58	10.3	36
4	10.39	23.4	89	29	23.84	12.2	35
5	11.81	19.4	77	30	23.92	12.4	36
6	14.12	18.9	68	32	24.18	11.5	36
7	16.77	15.0	37	34	24.18	12.2	36
8	17.99	13.9	36	36	24.65	11.2	36
9	18.78	13.4	36	38	24.80	12.3	36
10	19.38	14.3	36	40	25.03	11.9	35
11	20.04	12.6	36	42	25.07	13.1	35
12	20.31	12.6	36	44	25.52	15.3	34
13	21.04	13.0	36	46	25.68	14.6	33
14	21.21	13.2	36	48	25.45	14.6	32
15	21.78	13.7	36	50	25.50	14.0	32
16	22.14	12.6	36	52	25.76	15.3	29
17	22.29	11.9	36	54	25.78	15.8	29
18	22.22	11.9	36	56	26.00	16.5	29
19	22.60	12.0	36	58	26.26	16.7	29
20	22.60	11.2	36	60	26.12	17.8	29

growth of the animals is still occurring, though with extreme slow-
ness, even between the 50th and 60th weeks succeeding birth.

In females the succession of events is very similar, save that
the second cycle merges more gradually into the third, which
begins to exert a noticeable effect upon the growth of the animal
at a somewhat earlier age than in the male.

All the cycles are less extensive in the female than the male,

the growth curve of the female lying completely under that of
the male.   The difference in favor of the male at the end of Cycle
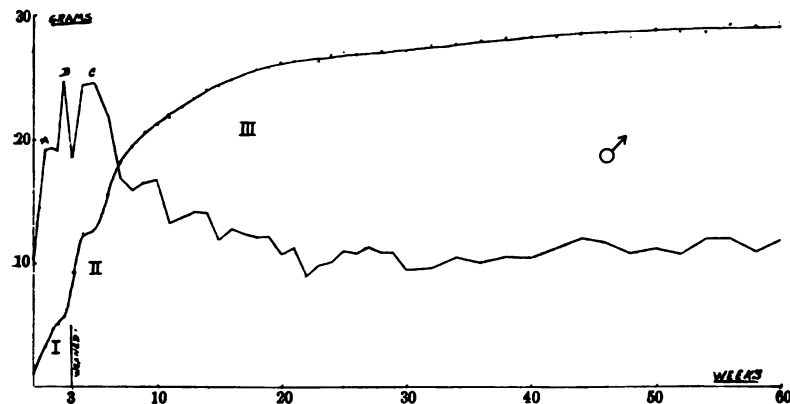
Fig. 1. Changes in weight (smooth curve) and variability (irregular
curve) of normal male mice with age.   Ordinates of the growth curve, gm.;
the dots represent actual observations.   Ordinates of the variability curve
represent percentages.
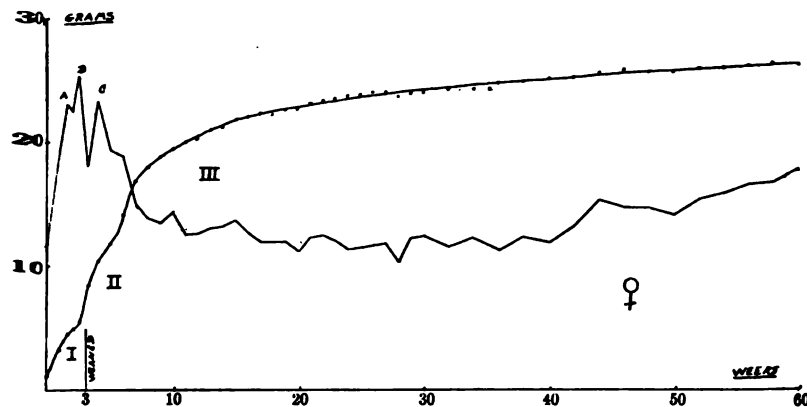
Fig. 2. Changes in weight (smooth curve) and variability (irregular
curve) of normal female mice with age.   Ordinates of the growth curve,
gm.; the dots represent actual observations.   Ordinates of the variability
curve represent percentages.

I (14 days) is 0.23 gm.; at the end of Cycle II (5 weeks) it is 0.64
gm.; and at 60 weeks it is 2.96 gm.

All the observed points lie almost exactly upon the smooth curves. But a slight tendency to depart therefrom is distinctly noticeable in both sexes at about the 25th week; very slight oscillations in the growth curve make their appearance here and afterwards disappear. It would appear that the age of about 25 weeks may be a period of relative instability in the growth of these animals.

The algebraic formulation of these curves will be deferred to a subsequent article of this series.

The variability curves display an unmistakable tendency of the variability to increase with increasing velocity of growth,
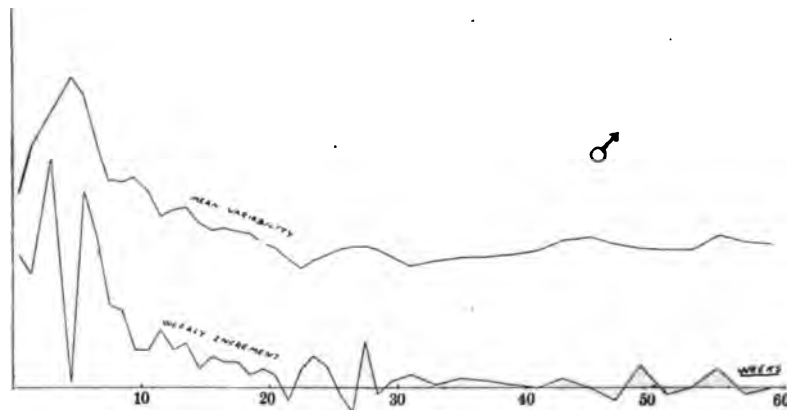
Fig. 3. Comparison of the mean variability (per cent) and the weekly increment in weight (gm.) of normal male mice.

and to decrease with decreasing velocity of growth. Thus in each sex the variability curve displays three maxima, marked A, B, and C, corresponding more or less closely in time with the three maxima of growth velocity due to Cycles I, II, and III, and thereafter the variability curve gradually falls to a relatively steady minimum value as the velocity of growth gradually decreases.

This relationship of variability to velocity of growth is clearly displayed in Figs. 3 and 4 in which the curve of mean variability is compared with the curve of weekly increments for males and females respectively. In constructing these curves the increment

from the end of the 5th to the end of the 6th weeks, for example, is regarded as the rate of weekly increment at the middle of the 6th week, while the mean of the variabilities at the end of the 5th and 6th weeks is regarded as the mean variability at the middle of the 6th week. It will be seen that the two curves run closely parallel to one another; in other words, the changes in variability are approximately proportional to the changes in weekly increment.

It will be noticed, however, that there is a distinct tendency for the variability of both sexes, but especially of the females, to undergo a progressive increase after the 40th week, without
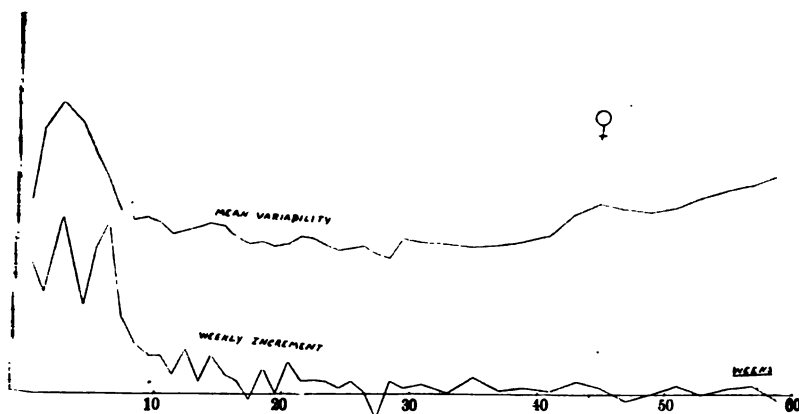
Fig. 4. Comparison of the mean variability (per cent) and the weekly increment in weight (gm.) of normal female mice.

any corresponding increase in the magnitude of the weekly increment.

Confirmation of Daniel's Estimate of the Period of Lactation.

It will be observed, on examining the growth-curves in Figs. 1 and 2, in which the end of the period of lactation is indicated by a vertical line, that the pause in growth which accompanies the culmination of Cycle I precedes by a distinct interval (1 week) the time at which the animals are weaned. Therefore this pause is not due to insufficient nourishment following the cessation of the supply of mother's milk. Furthermore the acceleration due to

the initial stages of the second cycle is already well under way before weaning occurs, and is therefore not attributable to the greater variety of diet enforced on the animals by the deprivation of milk.   In fact the weaning of the animals is accompanied by not the slightest distortion of the growth curve which passes smoothly and with a steep slope through the point corresponding to the date of weaning.   It is clear, therefore, that weaning on the 21st day after birth results in no physiological disturbance whatever in the young.

Estimate of the Number of Animals Required in Growth Experiments.

From the estimates of variability in weight at different ages which are tabulated above, it is possible to estimate the number of animals of a given sex and age which must necessarily be employed to obtain a measure of average weight which shall possess any desired degree of accuracy.

The *probable error* ϵ in the estimate of the mean, that is, the error which is as likely as not to occur, is expressed by:

$$\frac{0.6745\,\sigma}{\sqrt{N}} = \epsilon$$

where σ is the standard deviation estimated in the manner described above, and N is the number of measurements made; *i.e.*, animals weighed.[7] The variabilities tabulated above are $\frac{\sigma}{W} \times 100$ where W is the mean weight for the given age and sex.

Now the variability in weight of normal male mice at 40 weeks of age is about 10 per cent, and the mean weight is 28.3 gm. Hence,

$$\sigma = 2.83 \text{ and } \epsilon = \frac{0.6745 \times 2.83}{\sqrt{N}}.$$

If it is desired to make ϵ of any required dimensions, for instance 1 per cent, we can insert this value of ϵ in the formula and calculate therefrom N or the number of animals which we must employ in order to obtain an estimate of the mean weight which will probably lie within 1 per cent of the true value.

In the present instance, if ϵ is 1 per cent of the mean weight of the animals at 40 weeks we have:

$$\frac{0.6745 \times 2.83}{\sqrt{N}} = 0.283$$

whence $N = 45$. In other words, we see that in order to obtain an estimate of the mean weight of males at 40 weeks of age which shall as likely as not lie within 1 per cent of the true value, we must weigh no fewer than 45 animals.

In order to obtain an estimate of the mean weight which shall just as likely as not lie within 2 per cent of the true value, we must weigh 14 animals.

The variability of normal female mice at 40 weeks of age is about 12 per cent, and their mean weight 25 gm., from which we find that in order to obtain an estimate of the mean weight of females at 40 weeks of age which shall just as likely as not lie within 1 per cent of the true value we must employ 65 animals. If we are content with an estimate which may differ by 2 per cent from the true value we may employ 16 animals.

At 5 weeks the variability of males is about 25 per cent and the mean weight 12.45 gm. Hence the number of animals required to obtain an estimate of weight subject to a probable error of 1 per cent at this age is no less than 282. If we are content with a probable error of 2 per cent we may employ 71 animals.

For females at 5 weeks (variability, 20 per cent; mean weight, 10.39 gm.) the corresponding numbers are 183 and 46.

In the measurements tabulated above, and in general throughout the growth experiments to be described in this series, the number of animals of one sex and experimental class employed was, with few exceptions, intermediate between the number necessary to secure a probable accuracy of 1 per cent and that necessary to secure a probable accuracy of 2 per cent.

In the preceding article on methods it is shown that the probable error in individual weighings due to the fluctuating contents of the alimentary canal, and also that due to the weighings (subsequent to 4 weeks of age) being expressed to the nearest 0.5 gm., are approximately of the same magnitude; namely, from 1 to 2 per cent.

From these estimates it may be clearly seen how little reliance may be placed upon experimental conclusions concerning growth, published in not a few quarters, which are based on measurements made upon 2 to 6 animals.

External Changes in Mice Which Accompany Their Growth.

According to Daniel,[5] "Between birth and maturity four well defined stages occur. . . . The *first stage* is that in which the newly born young have a peculiarly red and transparent skin through which is seen the stomach white with milk. Following this at the end of the sixth or seventh day a *second stage* is evident in which the body is covered with flaky scales of dandruff,—forerunners of a coat of silky fur. A *third important stage.* . . . is usually shown on the ninth or tenth day at which time the mammæ in the young females appear. These can be observed for an interval up to the thirteenth or fourteenth day, at which time the fur usually obscures them. . . . The *fourth period*, on the fourteenth day, is characterized by the advent of sight. . . . The regularity with which this period occurs. . . . is a sufficiently exact criterion to make it an index of age."

I can add my testimony to the above as to the extraordinary invariability of the advent of sight on the 14th day after birth. Unusually small and unusually large animals open their eyes upon the same day. In a very few cases I have observed the eyes to open on the 13th day and in equally few cases not until the 15th, but in the great majority of cases the eyes open precisely on the 14th day after birth. It will be noted that this phenomenon, together with the attainment of a full coat of fur, coincides exactly with the termination of the first cycle of growth.

Birth occurs during the first half of the first growth cycle, exactly as it does in human beings.[9]

The vagina opens at about the 6th week and individuals may occasionally pair at this age, although usually not until later. The attainment of puberty, therefore, coincides with the period of maximum velocity of growth in the third growth cycle, exactly as it does in human beings.[10]

[10] Robertson, *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 581; *Am. J. Physiol.*, 1915, xxxvii, 1.

Changes in the Thyroid and Thymus Which Accompany Growth.

The anterior lobe of the pituitary body, the pineal body, the thyroid, and the thymus are known to exert notable influences upon the growth of animals. Their development in relation to the development of the animal as a whole is therefore of great interest. The pituitary and pineal bodies in such small animals as mice are inaccessible to measurement, but the thyroid and the thymus may readily be dissected out and weighed. This was accordingly done at certain definitely selected ages: at birth (10 individuals without distinction of sex); at 14 days, *i.e.*, at the culmination of the first growth cycle (10 males, 11 females); at 35 days, *i.e.*, at the culmination of the second growth cycle (10 males, 10 females); at 70 days (10 males, 10 females); and at 210 days (6 males, 6 females).

TABLE III.

Absolute Weights of Thyroid and Thymus.

Age.	Thyroid. ♂	Thymus. ♂	Thyroid. ♀	Thymus. ♀
	gm.	*gm.*	*gm.*	*gm.*
At birth..............	0.006	0.004	0.006	0.004
14 days..............	0.032	0.030	0.031	0.029
35 "	0.112	0.061	0.095	0.071
70 "	0.187	0.049	0.137	0.064
210 . "	0.208	0.031	0.179	0.045

The animals for this purpose were selected at random from our general stock and had been fed and treated in exactly the same way as other normal animals. At the required ages they were weighed and then chloroformed. They were then dissected and the thyroid and thymus immediately weighed to the nearest mg.

The thyroid in the mouse is a large triangular shaped organ, the apex of the triangle being directed ventrally and posteriorly, and the two extremities of the base being surmounted by small hemispherical elevations differing somewhat in appearance from the rest of the gland. A longitudinal furrow indistinctly divides the gland into right and left lobes.

The thymus is a distinctly lobated organ situated in the thoracic cavity just ventral and somewhat anterior to the heart.

Table III summarizes the observed absolute weights of these

glands in males and females at the ages enumerated, the figure
for the weights at birth referring to males and females withou
distinction of sex.

It will be observed that during the first cycle of growth th
thyroid and thymus both increase considerably in weight and t
about the same extent. During the second cycle the thyroi
increases much faster than the thymus. After the culminatio
of the second cycle of growth the thymus decreases markedl
in weight in both sexes, while the thyroid continues to grow. U
to the culmination of the first cycle of growth the thyroid an
thymus are practically of equal weight in both sexes, but subse
quently to this the thyroid grows more rapidly in the male tha

TABLE IV.

Weights of Thyroid and Thymus in Relation to Body Weight.

Age.	Thyroid. ♂	Thymus. ♂	Thyroid. ♀	Thymus. ♀
	per cent	*per cent*	*per cen'*	*per cent*
At birth.............	0.47	0.30	0.47	0.30
14 days	0.71	0.68	0.71	0.65
35 "	0.78	0.45	0.79	0.60
70 "	0.90	0.24	0.73	0.34
210 "	0.77	0.12	0.80	0.20

in the female, while the thymus grows more rapidly in the femal
than in the male.

In Table IV the weights of the thyroid and thymus are e>
pressed in mean percentages of the total body weights of th
individuals from which they were taken.

It will be observed that during the first cycle of growth th
thyroid and thymus both increase more rapidly than the bod
weight. Subsequently to this the thyroid increases almost i
direct proportion to the body weight, while the thymus increase
less rapidly than the body weight, or decreases.

The development of these glands therefore presents well marke
stages which coincide closely with the cycles displayed in th
growth curve of the animals.

Changes in the Alcohol-Soluble Phospholipoid Content of the Tissues during Growth.

Several investigations to which reference has already been made having shown that the phospholipoids and also certain other alcohol-soluble phosphorus-containing substances[11] exert a decided influence upon certain types of growth, an attempt was made to ascertain the changes in the content of this type of substances in the tissues of the mouse which accompany growth. For this purpose the animals used in estimating the weights of the thyroid and thymus were employed, the glands being restored to the carcases after weighing. The animals were decapitated, it being thought that the extensive phospholipoid content of the cerebral tissues, where lipoids perform the unusual function of constituting structural elements, would confuse the relationship between the lipoid content and the stage of development which might conceivably subsist in other tissues. The decapitated carcases were minced twice over in a small mincing machine, the minced tissue was weighed and then transferred quantitatively to a mortar in which it was thoroughly ground up with three times its weight of a mixture of equal parts by weight of anhydrous sodium and calcium sulfates. This mixture was then quantitatively transferred to a shallow dish and dried on a water bath. It was stirred from time to time to prevent caking. Drying under these conditions[12] is very rapid, being complete in about 1 hour. The dried mass was then transferred again to the mortar and thoroughly pulverized.

This pulverized material was quantitatively transferred to alundum extraction thimbles 30 mm. in diameter and 54 mm. high, which were placed in the flasks of a Bailey-Walker extraction apparatus,[13] the flasks being 1 inch longer than in the model usually supplied.[14] The alundum thimbles were made by cutting down the ordinary 80 by 30 mm. type to 54 mm. in length, drilling three small holes near to the top, and twisting into these small lengths of aluminum wire, which projected outwards and by contact with the neck of the flask held the thimbles upright,

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[11] Compare article No. IV in this series.
[12] Compare Leathes, J. B., The Fats, London, 1910, 53.
[13] Walker, P. H., and Bailey, L. H., *J. Ind. and Eng. Chem.*, 1914, vi, 497.
[14] By Eimer and Amend, New York.

the bottom of the thimbles resting on the indentations at the bottom of the neck of the flask and just over the boiling solvent employed in the extraction.   This apparatus has the double advantage of carrying out the extraction at the boiling temperature of the solvent, and of very rapid and simple manipulation.

The material was extracted continuously with boiling alcohol for 48 hours.   The combined alcoholic extracts from the material derived from 10 mice (11 in the case of 14 day females, 6 each in the cases of 210 day males and females) were measured and an

TABLE V.

Alcohol-Soluble Phosphorus in Gm. per 100,000 Gm. of Fresh Tissue.

Age.	♂	♀
At birth. *days*	$\frac{91}{96}$ Mean = 94	$\frac{91}{96}$ Mean = 94
14	92	$\frac{90}{94}$ Mean = 92
35	$\frac{88}{85}$ Mean = 87	$\frac{91}{88}$ Mean = 90
70	77	$\frac{79}{80}$ Mean = 80
210	$\frac{62}{57}$ Mean = 60	$\frac{65}{66}$ Mean = 66

aliquot portion of each combined extract corresponding to 5 gm. of the freshly minced tissue (2.5 gm. in the case of the mice at birth) was evaporated by placing it in a Kjeldahl digestion flask and plunging the flask into boiling water to a depth exceeding that of the contained liquid.

The residue was ashed with Neumann's sulfuric-nitric acid mixture and the phosphorus determined by von Wendt's modification of Neumann's method.[15]   In each case, excepting the 14 and 70 day males, the estimations were made in duplicate. Table V shows the results obtained.

[15] Von Wendt, G., *Skandin. Arch. Physiol.*, 1905, xvii, 217.

It will be observed that there is a steady decrease in the alcohol-soluble phosphorus in the tissues of mice from birth onwards, and almost exactly in direct proportion to their age. It should be noted that were this rate of decrease to be maintained the alcohol-soluble phosphorus content of the tissues would fall to zero at 630 days; i.e., at 21 months after birth.

These results differ strikingly from those reported by Siwertzow[16] in human beings. This observer states that the alcohol-soluble phosphorus in certain specified organs undergoes marked fluctuations with age, increasing in infants up to the age of 2 years and thereafter decreasing markedly. It is possible that these results merely indicate a transfer of alcohol-soluble phosphorus from one organ to another, the tissues of the animal as a whole becoming continuously poorer in this substance. It is also possible that the P : N ratio in the alcohol extract might reveal fluctuations bearing some relation to the growth cycles. This question is being investigated and will form the subject of a subsequent communication.

Analysis of the Causes of Death.

Between the 10th and 60th weeks six deaths were recorded among 36 normal males[17] and 7 among 36 normal females. The following is a summary of the causes of death:

Males.

Injuries received in fighting.................................... 2
Accidental injuries... 2
Unknown causes.. 2
   Total.. 6

Females.

Injuries.. 1
Pneumonia.. 3
Cancer... 2
Unknown causes.. 1
   Total.. 7

[16] Siwertzow, D. I., Dissertation, St. Petersburg, 1904; cited after Biochem. Z., 1904, ii, 310.

[17] This excludes the six males in one compartment which simultaneously developed paratyphoid at the unusually advanced age of 17 weeks and were replaced. The weight data referring to these animals were utilised in Table I up to the 12th week and omitted thereafter.

SUMMARY.

1. The curves of growth and variability of the white mouse have been determined from birth to the end of the 60th week succeeding birth under conditions and with stock exactly resembling those employed in the experiments to be detailed in subsequent communications of this series.

2. The average number of animals born in one litter is 5.15. The variability of the number born in a litter is very high; namely, 42.8 per cent. 29 per cent of the animals born in the laboratory died from various causes before being weaned on the 21st day after birth.

3. In each sex there are three separate extra-uterine growth cycles. The first cycle attains its maximum velocity at some time shortly prior to 7 days after birth and culminates at 14 days. The second cycle attains its maximum velocity at 21 to 23 days and culminates soon after the 28th day. The third cycle attains its maximum velocity at about 6 weeks and thereafter decreases in velocity continuously but very slowly, so that growth of the animals still occurs between the 50th and 60th weeks succeeding birth.

4. All the cycles are less extensive in the female than in the male.

5. The variability in weight of the animals shows a decided tendency to increase with increasing velocity of growth and to decrease with decreasing velocity of growth. After the 40th week, however, especially in the females, there is a tendency of the variability to increase progressively without any corresponding increase in the rate of growth.

6. Weaning of the animals on the 21st day after birth produces no physiological disturbance whatever in the young.

7. It is shown from the variability of the weight that considerable numbers of animals must be employed to obtain reliable data in experiments upon growth. The weight data reported in this and succeeding articles are probably within 1 or 2 per cent of the true values.

8. Birth in mice occurs during the first half of the first growth cycle. The eyes open coincidently with the culmination of the first growth cycle, a complete coat of fur being acquired at the same time. Puberty coincides with the period of maximum velocity of growth due to the third growth cycle.

9. The development of the thyroid and of the thymus in mice presents well marked stages which coincide closely with the cycles displayed in the growth curve of these animals. Subsequent to the culmination of the first growth cycle the thyroid is heavier in the male than in the female, while the thymus is heavier in the female than in the male.

10. There is a steady decrease in the percentage content of alcohol-soluble phosphorus in the tissues (other than cerebral tissues) of mice from birth to 210 days of age, and this decrease is almost exactly in direct proportion to their age.

EXPERIMENTAL STUDIES ON GROWTH.

III. THE INFLUENCE OF THE ANTERIOR LOBE OF THE PITUITARY BODY UPON THE GROWTH OF THE WHITE MOUSE.

BY T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.)

Present Knowledge. Objects of the Experiments.

The almost unanimous experience of observers is to the effect that the administration of the pituitary body either by mouth or hypodermically to young animals causes retardation of their growth.[1] This retardation is due to the anterior lobe, since anterior lobe alone produces retardation,[2] while the posterior lobe alone does not produce this effect upon growth.[3]

The results obtained by Schäfer[4] with rats are of significance when viewed in the light of the experimental results about to be enumerated. Very small numbers of animals were employed (three or four in each group), but the general result was that the early effect of feeding young animals with pituitary tissue was to cause retardation of growth, while later, that is when administration had been delayed until the animals were half grown, no such retardation and even some acceleration was displayed. Looking to the gross effect of these administrations (only continued

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[1] Caselli, A., *Riv. sper. freniat.*, Reggio-Emilia, 1900, xxvi, 176, 486. Fodera, F. A., and Pittau, L., *Gazz. med. e chir.*, 1909, viii, 149. Cerletti, U., *Arch. ital. biol.*, 1907, xlvii, 123. Etienne, G., and Parisot, J., *Arch. méd. exp. et d'anat. path.*, 1908, xx, 423. Sandri, O., *Arch. ital. biol.*, 1909, li, 337. Crowe, S. J., Cushing, H., and Homans, J., *Bull. Johns Hopkins Hosp.*, 1910, xxi, 127, cited after Cushing, H., The Pituitary Body and Its Disorders, Philadelphia, 1912.

[2] Aldrich, T. B., *Am. J. Physiol.*, 1912, xxx, 352; 1912–13, xxxi, 94 (in dogs and rats). Wulzen, R., *Am. J. Physiol.*, 1914, xxxiv, 127 (in fowls).

[3] Aldrich, *Am. J. Physiol.*, 1912–13, xxxi, 94.

[4] Schäfer, E. A., *Quart. J. Exp. Physiol.*, 1912, v, 203.

for 3 months), Schäfer concludes that administration of pituitary tissue exerts little or no effect upon growth. Having regard to the fact, to which attention is drawn in the preceding article of this series, that growth catalysers may be expected, and in many instances have been shown to exert totally opposite effects upon growth at different stages of development or upon differing types of growth, we cannot accept this negative conclusion. Schäfer's results, although lacking the conclusiveness which larger numbers of animals would supply, testify to the truth of the opposite conclusion, and in confirmation of his results and of mine which are about to be enumerated, Dr. Wulzen informs me that a fowl which she continued to feed with anterior lobe for a longer period than that covered by the results enumerated in her article (5 or 6 months) finally displayed acceleration of growth and not only caught up with but surpassed the normals in weight. Moreover, Robertson and Burnett[5] have shown that hypodermic administrations of emulsified anterior lobe of the pituitary body cause marked acceleration of the growth of carcinomata in rats.

It will be clearly seen from the above brief review of the hitherto available experimental data upon the influence of the anterior lobe of the pituitary body upon growth, that the precise nature of this influence may be expected to be considerably affected by the stage of development of the animals to which the gland is administered. This view, indeed, finds ample confirmation in the diversity of the clinical manifestations of pituitary insufficiency, and the profound extent to which, as Cushing has exhaustively shown,[6] these manifestations are modified according to whether the pituitary insufficiency or hyperactivity are pre-adolescent or post-adolescent in origin.

It appeared, therefore, advisable to enhance our knowledge of the mode of action of the anterior lobe of the pituitary body upon growth by obtaining exact information as to the position on the normal curve of growth occupied by the periods of development which are retarded and accelerated respectively by administration of this gland. With this object in view the experiments which are about to be described were undertaken.

[5] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280.

[6] Cushing, H., The Pituitary Body and Its Disorders, Philadelphia. 1912.

Description of the Experiments.   Mode of Administering the Tissue.
Dose Administered.

Average healthy young mice, not less than 4 or more than 5 weeks of age, were chosen at random and without selection from our stock, transferred to the cages described in the first article of this series, and immediately, and thereafter until the age of 60 weeks, fed upon anterior lobe emulsion. 36 males and 36 females were employed for this purpose. In every respect other than in the administration of the tissue these animals were fed and handled exactly as were the normal animals. Their cages were adjacent to the cages containing the normal animals, the two groups of animals were nearly of the same age, and were always weighed at the same time of day (middle afternoon). Hence no possible factor other than the administration of the pituitary tissue could possibly account for the deviation of these animals from the norm. That the observed deviation was not merely due to the presence of meat in the diet, irrespective of its kind, is conclusively proved in the communication which immediately succeeds this, in which it is shown that a substance may be isolated from the anterior lobe of the pituitary body, which, when administered together with a normal meat-free diet, produces exactly the same type of deviation from the norm as that observed in the experiments at present under consideration.

Through the kindness of the Superintendent, Mr. Ralston B. Brown, to whose cooperation with myself and other members of our staff I desire to express our great indebtedness, we obtained a continuous supply of fresh ox pituitaries from the local slaughter house of the Oakland Meat and Packing Company. These were fed to the mice as a rule less than 48 hours after their extraction from the oxen, being kept upon ice for the greater part of the time meanwhile. They were stripped of their connective tissue capsules, and the anterior and posterior lobes separated, the parts connecting the two lobes being detached from the anterior lobes. The anterior lobes were then mashed and ground to a fine pulp between two ground glass surfaces, and this pulp was put into the beaten and strained yolks and whites of eggs in the proportion of one anterior lobe to 10 cc. of egg. This mixture was shaken, allowed to stand on ice over

night, then shaken again thoroughly, and administered to the mice the following morning. 5 cc. of the mixture were administered to each compartment containing 6 animals, so that one anterior lobe was shared among every 12 animals. Weighings of several hundred anterior lobes showed the average weight of one lobe to be 1.5 gm., so that the average dose of fresh pituitary

TABLE I.

Pituitary-Fed Male Mice.

Age.	Weight.		No. weighed. (Pituitary-fed.)	Age.	Weight.		No. weighed. (Pituitary-fed.)
	Normal.	Pituitary-fed.			Normal.	Pituitary-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	12.38	12.78	20	25	27.05	25.92	36
5	12.45	13.13	36	26	26.94	26.11	36
6	15.58	15.86	36	27	26.55	26.11	36
7	18.08	17.38	36	28	27.19	26.29	34
8	19.36	18.60	36	29	27.08	26.23	35
9	20.63	19.54	36	30	27.23	26.48	33
10	21.19	20.03	36	32	27.61	26.68	36
11	21.81	20.58	36	34	27.71	27.35	34
12	22.65	21.79	36	36	27.95	27.33	32
13	23.31	22.36	36	38	28.20	27.55	32
14	23.96	23.00	36	40	28.27	28.03	32
15	24.28	23.06	36	42	28.28	28.05	31
16	24.75	23.67	36	44	28.62	28.32	31
17	25.21	24.11	36	46	28.55	28.24	31
18	25.61	24.17	36	48	28.22	28.05	30
19	25.81	24.57	36	50	28.88	28.47	30
20	26.10	24.63	36	52	28.68	28.58	30
21	26.28	24.99	36	54	28.67	28.92	30
22	26.06	25.35	36	56	29.32	28.90	30
23	26.34	25.85	36	58	29.10	29.35	30
24	26.82	26.19	36	60	29.08	29.42	30

tissue administered to each animal was 0.125 gm. The egg mixture was greedily eaten by the animals of all experimental classes, so that the consumption of the tissue administered in this way was practically invariably complete, a few shreds of connective tissue being the utmost that was left undevoured on any occasion. It cannot, of course, be stated that each animal invariably received the same dose, but in the long run that was

probably not far from the truth. The proportionate dose decreased with age, for it was not increased as the animals grew heavier. It was also somewhat larger for the females than for the males, since the females are somewhat less heavy than the males.

RESULTS.

The weights of these and of normal animals of the same ages are compared in Tables I and II and graphically illustrated in

TABLE II.

Pituitary-Fed Female Mice.

Age.	Weight.		No. weighed. (Pituitary-fed.)	Age.	Weight.		No. weighed. (Pituitary-fed.)
	Normal.	Pituitary-fed.			Normal.	Pituitary-fed.	
wks.	gm.	gm.		wks.	gm.	gm.	
4	10.39	9.53	31	25	23.79	22.04	35
5	11.81	11.76	35	26	24.04	22.10	36
6	14.12	13.89	35	27	24.00	22.29	35
7	16.77	14.71	36	28	23.58	22.54	36
8	17.99	16.24	36	29	23.84	22.39	35
9	18.78	17.22	36	30	23.92	22.91	34
10	19.38	17.93	36	32	24.18	23.07	36
11	20.04	18.24	36	34	24.18	23.42	36
12	20.31	18.88	36	36	24.65	23.63	36
13	21.04	19.21	36	38	24.80	24.01	36
14	21.21	19.57	36	40	25.03	24.38	36
15	21.78	19.72	36	42	25.07	24.44	36
16	22.14	19.90	· 36	44	25.52	24.62	34
17	22.29	20.49	36	46	25.68	24.68	34
18	22.22	20.72	36	48	25.45	24.74	34
19	22.60	21.16	35	50	25.50	25.38	34
20	22.60	21.21	36	52	25.76	25.71	34
21	23.06	21.34	35	54	25.78	25.82	33
22	23.32	21.67	36	56	26.00	26.06	33
23	23.51	21.67	35	58	26.26	26.26	33
24	23.68	22.07	36	60	26.12	26.65	31

Figs. 1 and 2, the undotted curves representing the normal growth of white mice during the experimental period, the dotted curves the growth of the animals which received the pituitary tissue. The dots represent the positions of the actual observations.

The effect of the administration is qualitatively the same in both sexes, but quantitatively greater in the females. Between the 6th and the 20th weeks growth is markedly retarded, so much so that at 20 weeks of age the pituitary males lag 1.47 gm., and the females 1.39 gm. behind the normals. Between the 20th and the 60th weeks, however, the growth of the pituitary-fed animals is markedly accelerated, so that they not only catch
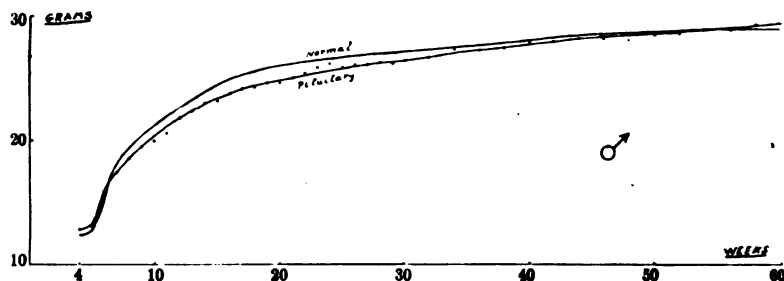
FIG. 1. Comparison of the growth curves of normal and of pituitary-fed males.
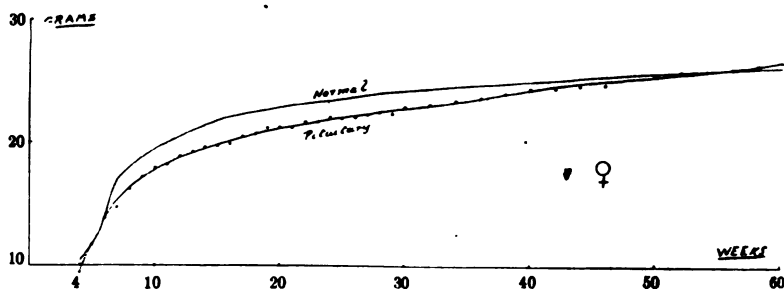
FIG. 2. Comparison of the growth curves of normal and of pituitary-fed females.

up to the normals, but actually, at about 1 year of age, come to surpass the normals in weight.

Summing up the effect of the anterior lobe of the pituitary body upon the rate of growth of mice subsequent to the 4th or 5th week after birth (culmination of the second growth cycle), it may ˙ ¯¯ he said that the earlier portion of the third growth ʰⁱˡ⁰ the latter portion is accelerated. The

that of other catalysers of growth.[7] Regarding the action of the anterior lobe of the pituitary body upon the first and second growth cycles, these experiments afford no information.

The variabilities of the pituitary-fed and of the normal animals are compared in Tables III and IV and illustrated graphically in Figs. 3 and 4. It will be seen that the effect of the adminis-

TABLE III..

Pituitary-Fed Male Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Pituitary-fed.		Normal.	Pituitary-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
4	24.4	22.7	25	11.0	9.8
5	24.6	24.1	26	10.8	9.2
6	22.0	22.1	27	11.3	8.8
7	16.9	17.7	28	10.9	8.7
8	15.9	17.3	29	10.9	8.8
9	16.5	14.8	30	9.5	8.4
10	16.7	15.4	32	9.6	9.0
11	13.3	14.9	34	10.5	9.7
12	13.7	11.4	36	10.1	9.3
13	14.2	10.7	38	10.5	9.3
14	14.1	10.0	40	10.4	9.2
15	11.9	9.8	42	11.2	10.6
16	12.7	9.3	44	12.0	10.0
17	12.4	9.9	46	11.7	10.1
18	12.1	9.7	48	10.8	11.1
19	12.1	10.2	50	11.1	11.2
20	10.8	9.8	52	10.7	11.2
21	11.2	9.6	54	12.0	11.4
22	9.0	11.5	56	12.0	11.1
23	9.8	8.7	58	10.9	11.0
24	10.1	10.5	60	11.8	11.0

tration is in both sexes to diminish the variability of the animals, especially between the 10th and the 20th weeks, when the difference in weight of the two groups of animals is also most marked. Between the 20th and 60th weeks, however, the variability curves of both sexes tend to approach the variability curves

[7] Compare the references cited in the early part of the second article of this series. '

ɔf the normals, and at about the 40th or 50th week the two va
bility curves intersect. The effect of the administration u
the variability of the animals is therefore similar to its effect u
the velocity of growth, a phenomenon which finds its explanat
in the fact, to which attention is drawn in the preceding art
of this series, that the variability of weight increases and decreɛ

TABLE IV.

Pituitary-Fed Female Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Pituitary-fed.		Normal.	Pituitary
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cen*
4	23.4	20.5	25	11.4	10.4
5	19.4	19.6	26	11.6	10.5
6	18.9	16.1	27	11.8	10.3
7	15.0	15.8	28	10.3	10.0
8	13.9	15.1	29	12.2	10.3
9	13.4	13.1	30	12.4	9.6
10	14.3	12.1	32	11.5	10.8
11	12.6	10.5	34	12.2	10.8
12	12.6	9.3	36	11.2	10.ſ
13	13.0	10.3	38	12.3	12.'
14	13.2	9.4	40	11.9	11.
15	13.7	10.4	42	13.1	12
16	12.6	9.3	44	15.3	13
17	11.9	9.6	46	14.6	1ſ
18	11.9	9.7	48	14.6	1'
19	12.0	9.7	50	14.0	1
20	11.2	9.1	52	15.3	
21	12.3	9.6	54	15.8	
22	12.5	10.2	56	16.5	
23	12.0	9.9	58	16.7	
24	11.3	10.2	60	17.8	

as the velocity of growth increases and decreases. Th
of growth of the pituitary-fed animals in the earlier
the third growth cycle being less than that of the nc
variability is also less, and as the relative velocity
increases so does the variability of weight.

It will be observed that the "period of relative inƒ

the age of about 25 weeks, to which attention was drawn in the preceding article, is also displayed plainly and at the same age in
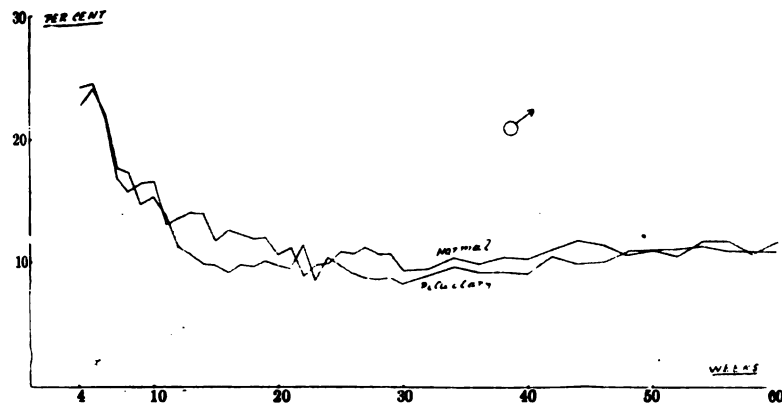
FIG. 3. Comparison of the variability curves of normal and of pituitary-fed males.
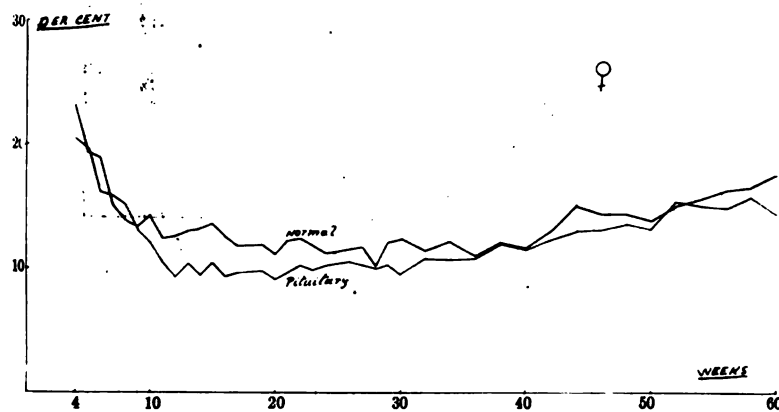
FIG. 4. Comparison of the variability curves of normal and of pituitary-fed females.

the pituitary-fed males by a tendency of the growth curve to oscillate at this point. In the pituitary-fed females this tendency is much less marked.

*Differences in the External Appearance and Behavior of the Pitui-
tary-Fed and the Normal Animals.*

From about the 30th week onwards it was noticeable that the
pituitary-fed animals were more compact or stocky in build than
the normals, and this divergence in degree of compactness in-
creased progressively. The pituitary-fed animals, weight for
weight, are smaller than the normals, and size for size they are
heavier. In the terminology employed by anthropologists the
ponderal index of pituitary-fed animals is higher than that of
normal animals.

Fig. 5. On the left a normal male 409 days old and 30 gm. in weight,
and on the right a pituitary-fed male 396 days old and 37 gm. in weight.
It will be noted that the pituitary-fed mouse is of approximately the same
linear dimensions as the normal, yet it is nearly 25 per cent heavier and
of a distinctly sturdier and more compact build.

This difference is clearly seen in the accompanying photograph
(Fig. 5) in which are shown a normal male 409 days old and
30 gm. in weight on the left, and a pituitary-fed male 396 days
old and 37 gm. in weight on the right. It will be noted that
the pituitary-fed mouse is of approximately the same linear dimen-
sions as the normal, yet it is nearly 25 per cent heavier and is
of a distinctly sturdier and more compact build.

The general impression created by the appearance of these animals was that during the earlier growth of the pituitary-fed animals the skeletal framework which was formed was smaller than that of the normal animals.[3] Subsequent to the hardening of the epiphyses these animals began to grow more rapidly than the normals and the accruing weight was, therefore, of necessity packed into a smaller space.

The male pituitary-fed mice also showed much more pronouncedly belligerent propensities than the normals. Normal male mice are sufficiently quarrelsome when confined together, but the pituitary-fed mice surpassed all records in this respect. Many of the males which were otherwise in normal health were in a continually lacerated condition. It was observed that the wounds healed quickly, and that infected wounds were rare.

Analysis of the Causes of Death.

Between the 10th and 60th weeks six deaths were recorded among the pituitary-fed males and three among the pituitary-fed females. The following is a summary of the causes of death:

Males.
Injuries received in fighting.................................. 6

Females.
Cancer.. 2
Unknown causes.. . . . 1

Comparing the above figures with the two deaths due to injuries received in fighting which were recorded among a similar number of normal males in the same time, a striking quantitative measure of the increased belligerency of pituitary-fed males is obtained.

SUMMARY.

1. The administration of 0.125 gm. per day per animal of fresh anterior lobe pituitary tissue to mice, beginning at 4 weeks after birth (conclusion of the second growth cycle) leads to retardation of growth during the earlier portion of the third growth cycle,

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[3] This is confirmatory of previous findings by Cerletti, Sandri, and Étienne and Parisot, referred to above.

between the 6th and the 20th weeks.   In the latter part of the third growth cycle, however, from the 20th to the 60th weeks after birth, the growth of the pituitary-fed animals is markedly accelerated, so that they not only catch up to the normals, but actually, at about 1 year of age, come to surpass the normals in weight.

2. The effect of the pituitary tissue upon the variability of the weight of the animals is similar to its effect upon the velocity of their growth.   The variability is diminished in the earlier portion of the third growth cycle, particularly between the 10th and the 20th weeks.   Between the 20th and the 60th weeks, however, the variability curves of the pituitary-fed animals approach the variability curves of the normals, and at about the 40th or 50th week the two variability curves intersect.

3. Pituitary-fed animals, from about the 30th week onwards, appear more compactly built than normal animals.   The pituitary-fed animals, weight for weight, are smaller than the normals of the same age, and size for size they are heavier.

4. Pituitary-fed males are noticeably more belligerent in their habits than normal males.

EXPERIMENTAL STUDIES ON GROWTH.

IV. THE INFLUENCE OF TETHELIN, THE GROWTH-CONTROLLING PRINCIPLE OF THE ANTERIOR LOBE OF THE PITUITARY BODY, UPON THE GROWTH OF THE WHITE MOUSE.

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(From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.)

INTRODUCTION.

From the concentrated solution prepared by extracting the dried tissue of the anterior lobe of ox pituitaries with boiling alcohol and evaporating this solution until solid material begins to separate out on cooling, a substance is precipitated by the addition of one and one-half times its volume of dry ether. This substance, after being washed in large volumes of alcohol-ether mixture, may be dried and pulverized and is readily soluble in water. I have called it *tethelin* (τεθηλως, growing) because, as the following experimental data show, its administration to mice is accompanied by effects upon their growth which precisely reproduce the effects, described in the preceding article, which accompany the administration of the whole tissue of the anterior lobe of the pituitary body.

The chemical characteristics, physiological actions other than that upon growth, and details of the method of preparing this substance are fully described in the article which immediately succeeds this. The purpose of the present article is solely to describe the influence which its administration exerts upon the growth of white mice.

The yield of tethelin from 300 anterior lobes was found in a number of instances to lie between 2.6 and 3.0 gm. Taking the higher figure, thus making allowance for possibly incomplete yields, we find the average content of tethelin in each anterior lobe to be 10 mg.

Keeping in mind the possibility that the above estimate might be considerably below the true mark (since tethelin is extracted very slowly from the dried tissue even by boiling alcohol), I thought it well, in initiating these experiments, not to adopt it as a basis of dosage nor to attempt to give to the experimental animals amounts of tethelin equal to those presumably contained in the pituitary tissue supplied to the pituitary-fed animals. It was thought better to err on the side of safety and consequently the amount administered was 50 mg. per 12 mice, corresponding, if the above estimate is nearly correct, as I now believe it to be, to no less than five anterior lobes per day per 12 mice. To the pituitary-fed animals only one anterior lobe was administered per day to 12 mice. Consequently the amount of tethelin received by these animals was nearly five times that which was received by the pituitary-fed animals, a fact which must be carefully borne in mind when the quantitative aspects of the results are under consideration.

Owing to the large demands upon the available material which were made by the chemical investigation of this substance, it was not found possible to spare a sufficient amount of tethelin continually to supply a large number of animals with so large a daily dose.

Two separate experiments were performed. For the one 24 male animals were chosen at random from among our stock, and these received the full dose daily (excepting on Sundays) and without interruption from the age of 5 weeks until the age of 60 weeks. For the second experiment 24 females were similarly chosen. These received the same dosage, but intermittently, in three periods of 1 month's duration each; namely, from the end of the 4th until the end of the 8th, from the end of the 21st until the end of the 25th, and from the end of the 42nd until the end of the 46th weeks.

The tethelin was triturated in a small glass mortar with distilled water in sufficient amount to make a 5 per cent solution. It readily dissolved, forming a cloudy brown solution having a characteristic greasy odor reminiscent of that of brain tissue. Not more than 1 week's supply was made up at any one time, since the solution does not keep, and it was stored, when made up, in the ice chest. The mixed whites and yolks of eggs were

beaten and strained, and to 20 cc. of this mixture were added
2 cc. of the tethelin solution. 5 cc. of this mixture were placed
in each compartment containing 6 mice. As in all other cases
the egg was eagerly eaten by the mice so that all the tethelin
administered was consumed.

TABLE I.

Tethelin-Fed Male Mice.

Age.	Weight.		No. weighed. (Tethelin-fed.)	Age.	Weight.		No. weighed. (Tethelin-fed.)
	Normal.	Tethelin-fed.			Normal.	Tethelin-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
5	12.45	13.05	20	26	26.94	25 30	23
6	15.58	14.69	24	27	26.55	25.59	23
7	18.08	16.54	24	28	27.19	25.46	23
8	19.36	18.21	24	29	27.08	25.63	23
9	20.63	18.98	24	30	27.23	25.70	23
10	21.19	19.50	24	32	27.61	26.00	23
11	21.81	19.92	24	34	27.71	26.22	23
12	22.65	20.56	24	36	27.95	26.02	23
13	23.31	20.88	24	38	28.20	26.24	23
14	23.96	20.88	24	40	28.27	26.43	23
15	24.28	21.21	24	42	28.28	26.54	23
16	24.75	21.90	24	44	28.62	26.50	23
17	25.21	22.65	24	46	28.55	26.74	23
18	25.61	23.21	24	48	28.22	26.78	23
19	25.81	23.79	24	50	28.88	26.52	23
20	26.10	24.35	24	52	28.68	26.98	23
21	26.28	24.52	24	54	28.67	27.63	23
22	26.06	24.87	23	56	29.32	27.72	23
23	26.34	24.61	23	58	29.10	27.33	23
24	26.82	24.91	23	60	29.08	26.93	23
25	27.05	25.33	23				

In every respect other than in the administration of tethelin
these animals were fed and handled exactly as the normal and
the pituitary-fed animals were fed and handled. Their cages
were kept in the same room as and not far removed from those
containing the normal mice. The tethelin-fed males were only
slightly (from 2 to 4 weeks) younger than the normals, while
the tethelin-fed females were only from 2 to 3 months younger
than the normals. Their life and growth were therefore con-

temporary with the greater part of the life and growth of the normal animals. All the animals were invariably weighed at the same time of day (in the middle of the afternoon), so that no possible factor other than the administration of tethelin could account for the observed deviations of these animals from the norm.

TABLE II.

Tethelin-Fed Female Mice.

Age.	Weight.		No. weighed. (Tethelin-fed.)	Age.	Weight.		No. weighed. (Tethelin-fed.)
	Normal.	Tethelin-fed.			Normal.	Tethelin-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	10.39	10.96	12	25	23.79	21.75	16
5	11.81	13.44	16	26	24.04	22.00	16
6	14.12	14.94	16	27	24.00	21.84	16
7	16.77	15.78	16	28	23.58	22.22	16
8	17.99	16.19	16	29	23.84	22.36	14
9	18.78	16.63	16	30	23.92	22.38	16
10	19.38	17.03	16	32	24 18	22.59	16
11	20.04	17.53	16	34	24.18	23.06	16
12	20.31	17.66	16	36	24.65	22.88	16
13	21.04	18.97	16	38	24.80	23.44	16
14	21.21	19.72	16	40	25.03	23.53	16
15	21.78	20.53	16	42	25.07	23.53	16
16	22.14	20.19	16	44	25.52	23.94	16
17	22.29	20.59	16	46	25.68	23.59	16
18	22.22	20.31	16	48	25.45	23.50	16
19	22.60	20.59	16	50	25.50	24.09	16
20	22.60	20.59	16	52	25.76 ·	24.41	16
21	23.06	21.09	16	54	25.78	24.41	16
22	23.32	21.13	16	56	26.00	24.56	16
23	23.51	21.47	16	58	26.26	24.38	16
24	23.68	21.78	16	60	26.12	24.31	16

Results of the Experiments.

The weights of the tethelin-fed and of normal animals of the same ages are compared in Tables I and II and graphically illustrated in Figs. 1 and 2, the undotted curves representing the normal growth of white mice during the experimental period and the dotted curves the growth of the animals which received tethelin. The dots represent the position of the actual observa-

tions and, in the curve representing the growth of the tethelin-
fed females (Fig. 2), the heavily shaded portions of the base line
indicate the periods during which tethelin was administered to
these animals.

The results, it will be seen, are qualitatively identical with
those which accompany the administration of the whole anterior
lobe, as described in the preceding communication. From the
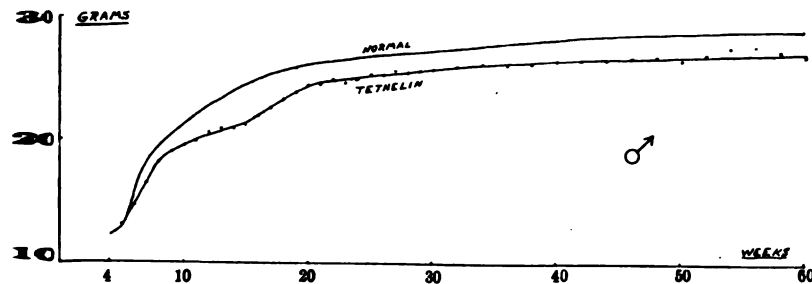
FIG. 1. Comparison of the growth curves of normal and of tethelin-
fed males.
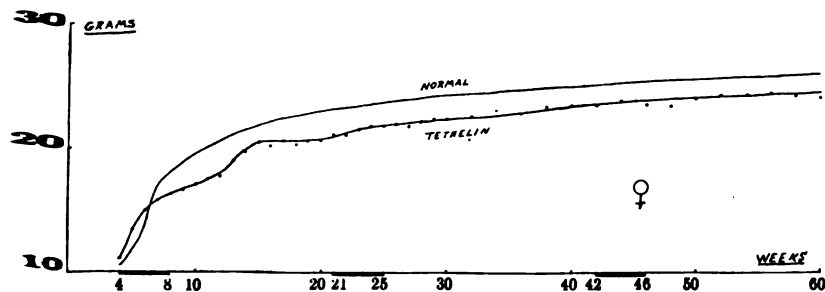
FIG. 2. Comparison of the growth curves of normal and of tethelin-
fed females. The heavily shaded portions of the base line indicate the
periods during which tethelin was administered to the animals.

5th until the 15th week the growth of the males is greatly re-
tarded. From the 15th week on marked acceleration is evi-
denced which continues until the 25th week, after which the
growth curve of the tethelin-fed animals remains nearly parallel
to and somewhat below that of the normals. These effects are
so marked as to cause complete distortion of the growth curve,
giving the appearance of great prolongation and enlargement of

the second growth cycle and acceleration and curtailment of the third growth cycle.

In females the effects observed are similar, but the retardation is less prolonged, being replaced by acceleration at the 12th week. The acceleration is not so prolonged and tends to disappear by the 20th week, when it is restored by the renewed administration of tethelin from the 21st to the 25th weeks. There-

TABLE III.

Tethelin-Fed Male Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Tethelin-fed.		Normal.	Tethelin-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
5	24.6	21.8	26	10.8	8.1
6	22.0	18.2	27	11.3	8.0
7	16.9	14.6	28	10.9	7.4
8	15.9	12.0	29	10.9	7.9
9	16.5	10.2	30	9.5	7.2
10	16.7	10.8	32	9.6	7.1
11	13.3	10.9	34	10.5	8.8
12	13.7	10.6	36	10.1	6.9
13	14.2	10.5	38	10.5	7.4
14	14.1	11.2	40	10.4	5.4
15	11.9	9.1	42	11.2	5.8
16	12.7	8.4	44	12.0	6.9
17	12.4	8.0	46	11.7	7.0
18	12.1	7.3	48	10.8	6.9
19	12.1	7.2	50	11.1	7.8
20	10.8	6.3	52	10.7	7.2
21	11.2	6.2	54	12.0	7.0
22	9.0	7.6	56	12.0	7.7
23	9.8	6.4	58	10.9	6.7
24	10.1	7.7	60	11.8	7.3
25	11.0	6.7			

after, with some oscillations, the growth curves of the tethelin-fed and of the normal animals remain nearly parallel.

Summing up the effect of the administration of tethelin upon the rate of growth of mice subsequent to the 4th or 5th week after birth (culmination of the second growth cycle), it may be said that the earlier portion of the third growth cycle is retarded, while the latter portion is accelerated. The differences observed

between the effects exerted by the whole anterior lobe of the pituitary body and those exerted by tethelin are purely quantitative and undoubtedly depend upon the different dosages employed in the two experiments. The milder dosage employed in the pituitary feeding experiments produced an initial retardation of the third growth cycle which was not irremediable, and hence the later acceleration of growth not only restored the weight of

TABLE IV.

Tethelin-Fed Female Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Tethelin-fed.		Normal.	Tethelin-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
4	23.4	27.6	25	11.4	12.8
5	19.4	19.6	26	11.6	13.5
6	18.9	19.4	27	11.8	13.6
7	15.0	16.1	28	10.3	14.2
8	13.9	12.6	29	12.2	14.1
9	13.4	12.4	30	12.4	15.4
10	14.3	13.2	32	11.5	15.4
11	12.6	15.7	34	12.2	15.7
12	12.6	16.6	36	11.2	15.7
13	13.0	14.1	38	12.3	15.6
14	13.2	11.9	40	11.9	16.9
15	13.7	11.4	42	13.1	17.9
16	12.6	11.2	44	15.3	18.8
17	11.9	11.5	46	14.6	17.8
18	11.9	10.7	48	14.6	17.1
19	12.0	11.9	50	14.0	17.6
20	11.2	12.1	52	15.3	16.6
21	12.3	11.2	54	15.8	17.2
22	12.5	11.1	56	16.5	17.1
23	12.0	12.2	58	16.7	16.7
24	11.3	12.7	60	17.8	16.8

the animals to normal but to a level slightly in excess of normal. The greater dosage employed in the tethelin feeding experiments led to so extreme an initial inhibition of growth that the lost ground could not be wholly made up by the subsequent marked acceleration of growth.

The variabilities of the tethelin-fed and of the normal animals are compared in Tables III and IV and illustrated graphically

in Figs. 3 and 4.[1] It will be seen that the effect of the adminis-
tration in the males is to diminish greatly their variability. The
effect of tethelin upon variability is therefore identical with the
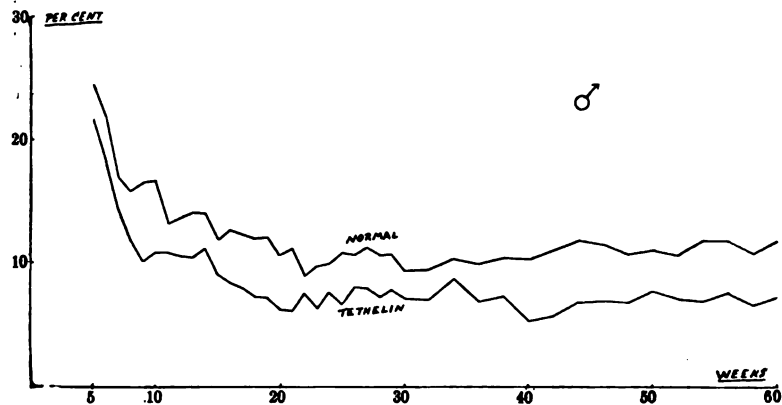
Fig. 3. Comparison of the variability curves of normal and of teth-
elin-fed males.
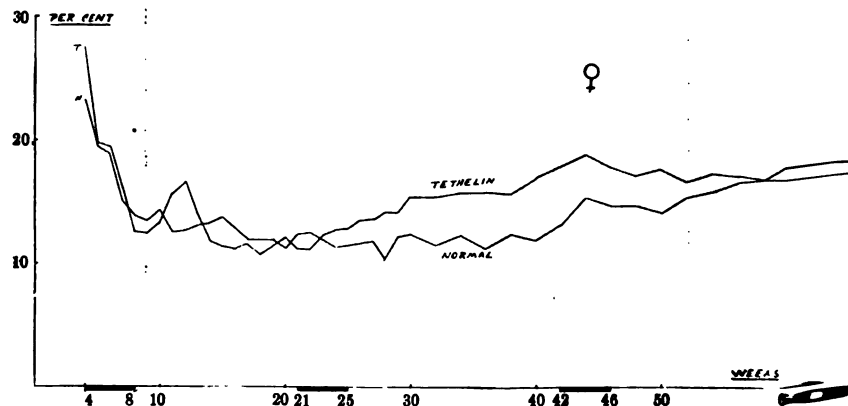
Fig. 4. Comparison of the variability curves of normal and of teth—
elin-fed females. The heavily shaded portions of the base line indicate
the periods during which tethelin was administered to the animals.

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[1] In Fig. 4 the variability curve of the tethelin-fed animals is slightly
distorted at 20 weeks by the omission of one measurement. This is done
to avoid intersection of the two curves at an angle and consequent con-
fusion.

effect of the anterior lobe of the pituitary body upon variability[2] and arises from the same cause; namely, the retardation of the rate of growth. In the females this would appear at first sight not to be the case, for the variability curve of the tethelin-fed females, after lying somewhat below that of the normals from the 8th to the 20th weeks, thereafter lies considerably above the variability curve of the normals. This is, however, due to the fact that the initial variability of the tethelin-fed group of females happened to be much greater than the initial variability of the normal group, and whether we diminish all of the observed variabilities of the tethelin-fed group by the amount of the initial excess, or express all the observed values as percentages of the initial variability, we obtain the same result; namely, that the variability of the tethelin-fed animals was diminished by the administration and strikingly so between the 8th and the 20th weeks.

Differences in the External Appearance and Behavior of the Tethelin-Fed and the Normal Animals.

The differences in build observed and commented upon in the preceding article between the pituitary-fed and normal animals were displayed in even more striking degree by the tethelin-fed animals, and particularly by the males. Their compactness of build is indeed remarkable. Weight for weight they are smaller and size for size much heavier than the normals. The contours of their surface are more rounded and youthful and, most remarkable of all, the coats of the males, even at 14 months of age, retain the glossy, silky appearance of the coats of young animals or of females, while 6 months or more prior to this age the coats of normal males are already shaggy, staring, and discolored.

These differences are clearly displayed in the accompanying photograph (Fig. 5) in which a normal and a tethelin-fed male of the same age (1 year) and of the same weight (28.0 gm.) are compared. The normal animal on the left has a shaggy, staring, and discolored coat, while the tethelin-fed animal has a smooth, glossy, and pure white coat. The normal animal is irregular in

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[2] Compare the preceding article of this series.

outline and loosely built, while the contour of the tethelin-fed animal is rounded and its build is compact and stocky.

It may here be mentioned that the improvement in the appearance of the coat which is so striking in the tethelin-fed animals was not observed in the pituitary-fed animals. Whether this difference was a matter of dosage merely, or due to the deleterious action of the meat contained in the dietary of the pituitary-fed animals upon their coats, I cannot say. Another and
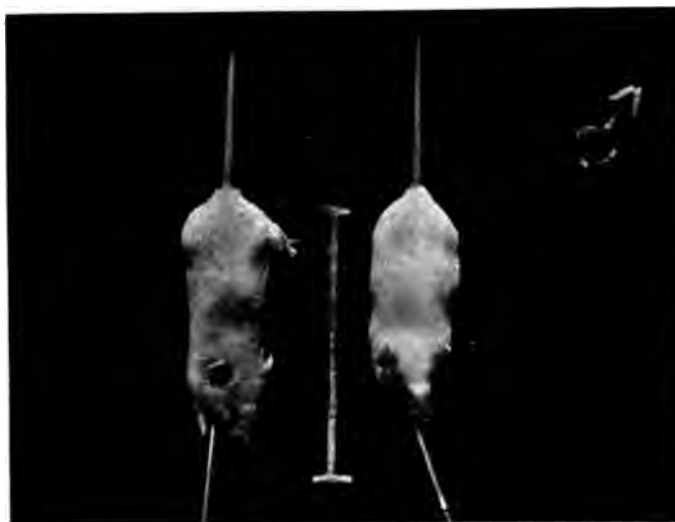
FIG. 5. Comparison of a normal (left) and a tethelin-fed male (right), both 1 year old and 28 gm. in weight. Note the smooth coat and compact form of the tethelin-fed mouse as contrasted with the loose form and rough coat of the normal animal.

striking difference was also noticed between the pituitary-fed and the tethelin-fed animals. The pituitary-fed males, as I pointed out in the preceding article, were remarkably and exceptionally belligerent in their behavior. The tethelin-fed males, on the contrary, were the quietest and least belligerent males in our collection. It would appear that the substance or substances in the tissue of the anterior lobe of the pituitary body which are responsible for the remarkable pugnacity which develops upon its admin-

istration to mice are not identical with or necessarily related to the growth-controlling constituents.

On reviewing these results there can remain little doubt that tethelin is really the growth-controlling constituent of the anterior lobe of the pituitary body. But lest any additional confirmation should be desired I may mention that Dr. Theodore C. Burnett and I have found that hypodermic administrations of tethelin to rats inoculated with carcinoma cause the same remarkable acceleration of the growth of the tumors which we have previously found to be the characteristic effect of similar administrations of the anterior lobe of the pituitary body.[3]

Analysis of the Causes of Death.

Between the 10th and the 60th weeks one death, from injury received in fighting, was recorded among the 24 tethelin-fed males, and one, from paratyphoid, among the 17 tethelin-fed females.

SUMMARY.

1. From the concentrated alcoholic extract of dried anterior lobes of ox pituitaries a substance may be precipitated by the addition of one and one-half volumes of dry ether. This substance I have termed *tethelin*.

2. The effects of tethelin upon the growth of white mice resemble in every particular the effects of the administration of the whole anterior lobe. These effects consist in marked retardation of the first portion of the third growth cycle followed by acceleration of the latter portion of the third growth cycle.

3. The variability in weight of the tethelin-fed animals, like that of the pituitary-fed animals, is less than that of normals of the same age.

4. Adult tethelin-fed animals, like adult animals which have been fed upon the anterior lobe of the pituitary body, are more compact in form and build than normal animals of the same age. Weight for weight the tethelin-fed animals are smaller than the normal ones, and size for size they are heavier.

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[3] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280.

5. The coats of adult male animals which have been fed tethelin retain, even at 14 months of age, the glossy, silk pearance of the coats of young animals. Normal males, e months prior to this age, have shaggy, staring, and disco coats. This improvement in the coat was not observed i pituitary-fed animals.

6. Tethelin-fed animals do not display the remarkabl hanced belligerency which is exhibited by pituitary-fed ani

ON THE ISOLATION AND PROPERTIES OF TETHELIN, THE GROWTH-CONTROLLING PRINCIPLE OF THE ANTERIOR LOBE OF THE PITUITARY BODY.

By T. BRAILSFORD ROBERTSON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.)

PLATE 1

(Received for publication, January 28, 1916.)

Method of Preparation.

Fresh ox pituitaries are stripped of their connective tissue capsules and the anterior lobes are separated from the posterior lobes and from the parts connecting the two lobes. The anterior lobes are then ground up in a mortar with three times their own weight of a mixture of equal parts by weight of anhydrous sodium and calcium sulfates[1] and the mixture is dried over a water bath. It is stirred occasionally to prevent caking. Drying under these conditions is extremely rapid and involves a minimum of exposure of the tissue to heated air. In the course of an hour a mixture of 60 gm. of tissue and 180 gm. of sodium and calcium sulfates becomes white and easily powdered. This dried mixture is returned to the mortar and pulverized as finely as possible and is then extracted with absolute alcohol at a temperature as near to that of boiling alcohol as possible.

For this purpose I employ a modified form (as described on page 379) of the Bailey-Walker extraction apparatus.[2] We obtain the apparatus from Eimer and Amend, New York. One of the extraction thimbles holds the dried powder derived from about six glands. 55 cc. of alcohol are employed in each flask.

In order to obtain a complete yield I find it necessary to carry on the extraction continuously for not less than 48 hours.

The mixed alcoholic extract obtained from 300 glands is filtered

[1] Compare Leathes, J. B., The Fats, London, 1910, 53.
[2] Walker, P. H., and Bailey, L. H., *J. Ind. and Eng. Chem.*, 1914, vi, 497.

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to remove the small amount of powder which has fallen into it during extraction, and is then evaporated under reduced pressure until reduced to a volume of about 800 cc., when solid material begins to separate out on cooling. When the concentrated extract is nearly cool it is placed in a tall narrow stoppered glass cylinder, mixed with one and one-half times its volume of dry ether, and the mixture is shaken. A flocculent precipitate appears in the mixture immediately and quickly settles out. After allowing this mixture to stand for several hours the supernatant liquid is siphoned off from the precipitate which is then suspended in somewhat over 2 liters of a mixture of one part by volume of absolute alcohol and one and one-half parts of dry ether and thoroughly shaken. After again allowing the precipitate to settle the supernatant fluid is siphoned off and the precipitate and residual fluid are transferred as quickly as possible to a thoroughly dry hardened filter paper in a thoroughly dry incubator over sulfuric acid, the temperature of the incubator being maintained at about 30–35°C. After a few minutes the incubator is momentarily opened to remove the alcohol-ether which has passed through the filter paper in the interim, and the filter and contained precipitate are allowed to dry for 24 to 48 hours. At the end of this period the dry substance, now in the form of small brittle and readily pulverizable cakes of a pale cream color, is transferred as rapidly as possible to a thoroughly dry glass container.[3]

The substance thus prepared would appear, from the constancy of its nitrogen and phosphorus content, to be a chemical unit. In the communication which immediately precedes this it has been shown that this substance exerts the same influence upon the growth of mice as the whole anterior lobe of the pituitary

[3] Better still, we may employ an apparatus which I have recently had constructed for this purpose, consisting of a large cylinder provided with a clamped lid, capable of withstanding an external pressure of one atmosphere, and provided with automatic electrical temperature control. Sulfuric acid is placed in the bottom of this, the filter funnel and a receptacle for drainage are placed on a false bottom above the acid, the lid is clamped on, and the cylinder is evacuated. The temperature being maintained at a point lying between 30 and 40°C., the ether and alcohol boil off, and when this is completed the vessel is allowed to remain evacuated at the above temperature for 24 hours.

ody. It would therefore appear to be the growth-controlling principle of the gland, and for this reason I have termed it *tethelin* τεθηλως, growing).

Properties.

Tethelin, prepared in the above manner, is a white or pale cream colored substance which is readily powdered. It rapidly absorbs aqueous vapor when exposed to damp air and becomes moist and darkens in color.

When heated it begins to darken at a temperature lying between 100 and 110°C. When heated to still higher temperatures the substance progressively darkens and softens as the temperature rises.

Tethelin is soluble in water to the extent of about 5 per cent, forming at that concentration a brown turbid solution. More dilute solutions are paler in color, and 1 per cent solutions are but slightly opalescent. Aqueous solutions of tethelin have a greasy odor, somewhat like that of brain tissue.

Tethelin is soluble in ethyl alcohol and, to a less extent, in ethyl ether, the solutions in ether being markedly opalescent at high dilutions. It is also soluble in chloroform and in carbon tetrachloride. It is insoluble in a mixture of one part by volume of absolute ethyl alcohol and one and one-half parts of dry ether.

Aqueous solutions of tethelin are faintly acid in reaction; 1 gm. the substance, dissolved in 200 cc. of water, requiring the addition of about 18×10^{-6} equivalents of sodium or potassium hydroxide (11 mg. of potassium hydroxide) to render the solution neutral to phenolphthalein.

The refractive index of a 1 per cent aqueous solution of tethelin exceeds that of distilled water at the same temperature by 0.00176.

Composition.

The phosphorus content of five separate samples from different preparations of tethelin was determined by von Wendt's modification of Neumann's method.[4] The precipitate of phosphomolybdic acid was dissolved in 0.1 N KOH and titrated to neu-

[4] Von Wendt, G., *Skandin. Arch. Physiol.*, 1905, xvii, 217.

trality to phenolphthalein by the addition of 0.1 N HCl, employing the factor: 1 cc. of 0.1 N KOH = 0.25357 mg. P_2O_5.[5]

The following were the results obtained:

0.230 gm. substance yielded 7.568 mg. P_2O_5, hence P = 1.44 per cent.
0.240 "   "   "   7.600 "   "   "   P = 1.38 "   "
0.195 "   "   "   6.238 "   "   "   P = 1.40 "   "
0.425 "   "   " 13.566 "   "   "   P = 1.39 "   "
0.410 "   "   " 13.312 "   "   "   P = 1.42 "   "

Mean content, P = 1.41 "   "

The nitrogen content of two samples from different preparations of tethelin was determined by the ordinary Kjeldahl method with the following results:

0.900 gm. substance yielded 0.02478 gm. N, hence N = 2.75 per cent.
0.760 "   "   "   0.02128 "   "   " N = 2.80 "   "

The nitrogen content in three other samples was determined by the Gunning-Arnold modification of the Kjeldahl method, with the following results:

0.418 gm. substance yielded 0.0108 gm. N, hence N = 2.58 per cent.
0.479 "   "   "   0.0116   "   "   "   N = 2.42 "   "
0.395 "   ".   "   0.0094   "   "   "   N = 2.38 "   "
          The mean of the five determinations is N = 2.58 "   "

These results yield the ratio P : N = 1 : 4.05, or, almost exactly, 1 : 4.

Amino Nitrogen Content.

The amino nitrogen content of three samples of tethelin was determined by the method of Van Slyke,[6] employing for each estimation 10 cc. of a 5 per cent solution. The following were the results:

0.50 gm. substance yielded 11.4 cc. moist nitrogen at 25° and 748 mm.
                              Hence N = 6.22 mg. = 1.24 per cent.
0.50 "   "   "   11.4 " moist nitrogen at 25° and 748 mm.
                              Hence N = 6.22 mg. = 1.24 per cent.
0.50 "   "   "   11.3 " moist nitrogen at 23.5° and 748 mm.
                              Hence N = 6.22 mg. = 1.24 per cent.

[5] Neumann, A., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1900, 163.

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.

The content of amino nitrogen is therefore 1.24 per cent, or almost exactly one-half of the total nitrogen. Since for every phosphorus atom there are four nitrogen atoms present in the substance, it is evident that two of these nitrogen atoms are present in amino groups.

Increase of Amino Nitrogen after Hydrolysis.

The molecule of tethelin also contains one imino group (per atom of phosphorus) which is converted into an amino group by hydrolysis as the following experiments show:

2.5 gm. of tethelin were triturated in 10 cc. of distilled water, 40 cc. of a saturated solution of barium hydroxide were added, and the mixture was boiled under a reflux condenser. At the end of 6 hours a 10 cc. sample, containing the soluble products of the hydrolysis of 0.50 gm. of tethelin, was placed in the Van Slyke apparatus.

Soluble products from 0.50 gm. substance yielded 15.1 cc. moist nitrogen at 23° and 750 mm.   Hence N = 8.31 mg. = 1.66 per cent.

It was inferred from this result that hydrolysis was still incomplete. Accordingly, to 30 cc. of the remainder of the solution were added 30 cc. of saturated barium hydroxide solution and the mixture was boiled for 16 hours. At the end of that period two 10 cc. samples were analyzed.

Soluble products from 0.25 gm. substance yielded 8.1 cc. moist nitrogen at 21° and 754 mm.   Hence N = 4.55 mg. = 1.82 per cent.
Soluble products from 0.25 gm. substance yielded 8.2 cc. moist nitrogen at 21° and 754 mm.   Hence N = 4.61 mg. = 1.84 per cent.

The mean of these estimates is 1.83 per cent, or nearly three-fourths of the total nitrogen content of tethelin.

Summarizing these results we may infer that tethelin contains four atoms of nitrogen for every atom of phosphorus, two of these atoms of nitrogen being contained in amino groups and one in an imino group which is converted into an amino group by hydrolysis with barium hydroxide.

Aqueous solutions of tethelin do not reduce Fehling's solution either before or after hydrolysis with barium hydroxide or barium hydroxide followed by sulfuric acid.

When tethelin is heated with strong sulfuric acid and sugar, no red or violet color is developed.

Aqueous solutions of tethelin give Ehrlich's reaction;[8] namely, a pink coloration on mixing the solution with an equal volume of a 2 per cent solution of p-dimethylaminobenzaldehyde in hydrochloric acid of specific gravity 1.09 and allowing the mixture to stand for a few minutes. This reaction is also given after prolonged boiling of the tethelin with barium hydroxide, but is no longer given after preliminary boiling in barium hydroxide solution followed by boiling in dilute sulfuric acid solution. According to Orgler and Neuberg,[9] this reaction indicates the presence of an acetylated oxy-amino acid radicle.

When chlorine is carefully added to an aqueous solution of tethelin, either before or after hydrolysis with barium hydroxide or barium hydroxide followed by sulfuric acid, a pink coloration appears in the mixture which is destroyed by a very slight excess of chlorine.

Aqueous solutions of tethelin, either before or after hydrolysis with barium hydroxide or with barium hydroxide followed by sulfuric acid, yield Wiedel's reaction as modified by Fischer;[10] namely, a red coloration when the solution is saturated with chlorine, boiled, evaporated to dryness, and the residue exposed to the vapors of ammonia, warmed, and allowed to stand. This reaction probably indicates the presence of an iminazolyl radicle, a possibility which becomes of great significance when viewed in the light of the fact that the physiologically active principles of the posterior lobe of the pituitary body are believed to be iminazolyl derivatives.[11]

[8] Ehrlich, P., *Med. Woch.*, 1901, i, 151. Pröscher, F., *Z. physiol. Chem.*, 1901, xxxi, 520. Müller, F., *Z. Biol.*, 1901, xlii, 561.

[9] Orgler, A., and Neuberg, C., *Z. physiol. Chem.*, 1903, xxxvii, 399.

[10] Fischer, E., *Ber. chem. Ges.*, 1897, xxx, 2226.

[11] Compare Barger, G., and Dale, H. H., *J. Physiol.*, 1910–11, xli, 499. Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1911, xliii, 182. Aldrich, T. B., *J. Am. Chem. Soc.*, 1915, xxxvii, 203.

Iodine Value.

The iodine value was determined in two samples of material by the method of Wijs.[7] In the first determination the sample was dissolved in 15 cc. of chloroform; in the second the sample was dissolved in 10 cc. of carbon tetrachloride. The following were the results:

0.317 gm. substance absorbed 103.9 mg. iodine; absorption value = 32.8 per cent.

0.257 " " " 85.97 " " " " = 33.5 per cent.

The mean absorption value was therefore 33.2 per cent.

It was observed that on allowing powdered tethelin to stand in contact with air containing traces of moisture for some weeks it became darker in color. The iodine value of a sample of substance which had been thus exposed to air for about 6 weeks was therefore determined with the following result (with 10 cc. of carbon tetrachloride as solvent):

0.295 gm. substance absorbed 64.48 mg. iodine; absorption value = 21.9 per cent. Hence the iodine absorption value of tethelin decreases markedly on standing in contact with air containing traces of moisture.

Reactions.

Tethelin, in aqueous solution, does not yield the biuret reaction. It yields an atypical reaction with Millon's reagent; that is, on the addition of a very small quantity of the reagent to an aqueous solution of tethelin a precipitate appears which turns pink on heating. This color is discharged by adding an excess of the reagent.

When tethelin in aqueous solution, either before or after saponification by barium hydroxide or boiling in barium hydroxide solution followed by boiling in dilute sulfuric acid solution ($\frac{N}{3}$), is boiled with nitric acid and the resulting mixture is evaporated to dryness, a yellow residue is left, which, on exposure to the vapors of ammonia, becomes a deeper yellow.

[7] Leathes, J. B., The Fats, London, 1910, 67.

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[8] Ehrlich, P., *Med. Woch.*, 1901, i, 151. Pröscher, F., *Z. physiol. Chem.*, 1901, xxxi, 520. Müller, F., *Z. Biol.*, 1901, xlii, 561.

[9] Orgler, A., and Neuberg, C., *Z. physiol. Chem.*, 1903, xxxvii, 399.

[10] Fischer, E., *Ber. chem. Ges.*, 1897, xxx, 2226.

[11] Compare Barger, G., and Dale, H. H., *J. Physiol.*, 1910–11, xli, 499. Dale, H. H., and Laidlaw, P. P., *J. Physiol.*, 1911, xliii, 182. Aldrich, T. B., *J. Am. Chem. Soc.*, 1915, xxxvii, 203.

Decomposition Products.

When several volumes of a saturated aqueous solution of barium hydroxide are added to a concentrated aqueous solution of tethelin, a bulky, flocculent precipitate appears in the mixture. This dissolves on heating, forming a yellow solution and simul- · taneously a granular precipitate. This precipitate resembles a barium soap of an unsaturated fatty acid, or a mixture of barium soaps, in its appearance, insolubility in water, and ability to blacken osmic acid when warmed.

After prolonged boiling under a reflux condenser and removal of the excess of barium hydroxide by means of carbon dioxide, the above solution is found to contain the following fractions.

A. A fraction which is precipitated, after concentration of the above solution by evaporating on a water bath and filtering off the barium carbonate, by the addition to the solution of several times its volume of absolute alcohol. This substance flocculates and settles quickly. When dry it is white, or slightly tinged with yellow, and absorbs moisture from damp air.[12] It is soluble in water and is precipitated from its aqueous solution by alcohol. It also yields a precipitate with lead acetate. It contains barium and phosphorus and also yields Scherer's test for *inosite;* i.e., a bright red coloration is developed when the substance is dissolved in nitric acid solution of specific gravity 1.2 and an equal volume of 10 per cent calcium chloride solution, and the same volume of 1 per cent platinic chloride solution are added and the mixture is evaporated to dryness in a porcelain dish and heated.[13] This fraction, when freed by repeated resolution and reprecipitation from the more slowly settling portion of the precipitate does not give Ehrlich's reaction with p-dimethylaminobenzaldehyde.

A determination of the barium content of this fraction, purified as far as possible by resolution in water and reprecipitation with

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[12] It is probable that two substances are precipitated by alcohol. The one precipitated in larger amount flocculates quickly, is white, non-hygroscopic, and not precipitable by lead acetate. The other, smaller in amount, settles more slowly, is yellow in color, hygroscopic, and precipitable by lead acetate.

[13] Salkowski, E., Z. *physiol. Chem.*, 1910, lxix, 478.

alcohol, washing in alcohol and ether, and drying over sulfuric acid, yielded the following result:

0.1568 gm. substance yielded 62.6 mg. BaSO$_4$. Hence Ba = 23.5 per cent.

A determination of the phosphorus content by von Wendt's modification of Neumann's method yielded the result:

0.0172 gm. substance yielded 2.21 mg. P$_2$O$_5$. Hence P = 5.6 per cent.

The ratio of Ba : P found in this fraction was therefore 1 : 1.06; in other words, barium and phosphorus are present in equivalent proportions.

B. This fraction is precipitated from the filtrate after removal of Fraction A by the addition of an equal volume of ether. This substance is soluble in water, does not contain either barium or phosphorus, yields Ehrlich's and Scherer's reactions, and is precipitated from its aqueous solution by the addition of an equal volume of a 25 per cent solution of lead acetate. The dried substance is lemon-yellow in color and very hygroscopic.

C. On evaporating the filtrate, after the separation of Fraction B to dryness, taking up as much of the residue as will dissolve in hot absolute alcohol, and adding to this alcoholic solution an equal volume of ether, a substance is precipitated which when dried is orange-yellow in color and very hygroscopic. This substance is soluble in water, yields Ehrlich's and Scherer's reactions and is not precipitated from its aqueous solution by the addition of an equal volume of 25 per cent lead acetate solution.

D. The portion of the residue which is insoluble in alcohol mentioned in the preparation of Fraction C, is grayish white when dried. It is soluble in water, insoluble in alcohol or ether does not yield Ehrlich's or Scherer's tests, and is not precipitated from its aqueous solution by the addition of an equal volume o 25 per cent lead acetate solution.

The mixed decomposition products of tethelin obtained by prolonged boiling in aqueous barium hydroxide solution followed by prolonged boiling in dilute sulfuric acid solution ($\frac{N}{3}$), after removal of the excess of sulfuric acid by means of barium hydroxide and removal of the excess of barium hydroxide by carbon dioxide, yield, upon the addition of lead acetate solution (25 per cent) a

precipitate which redissolves if too much lead acetate is added. After removal of this precipitate the mixture yields a further precipitate upon the addition of basic lead acetate solution. This latter precipitate, when suspended in water and treated with hydrogen sulfide, yields a substance which resembles *dl*-inosite in the following particulars:

It is soluble in water, tastes sweet, and is precipitated from its aqueous solution in the form of white acicular crystals by the addition of several volumes of ethyl alcohol followed by the addition of a sufficient volume of ethyl ether to render the mixture cloudy. A sample of these crystals, purified by resolution and reprecipitation, washed in alcohol and ether, and dried over sulfuric acid, melted with discoloration (in contact with air) at 219°C. (uncorrected).[14]

The aqueous solution of the crystals, when evaporated to dryness with a small quantity of mercuric nitrate, yields, upon heating the residue, a red color which disappears on cooling and reappears on heating.

When a few of the crystals are dissolved in a drop or two of nitric acid of specific gravity 1.2, an equal volume of a 10 per cent solution of calcium chloride, and the same volume of a 1 per cent solution of platinic chloride are added to the solution, this mixture is evaporated to dryness, and the residue heated, a rose-red color appears which disappears on cooling and reappears with a bluish tinge on reheating.

The presence of both phosphorus and inosite in the growth-controlling principle of the anterior lobe of the pituitary body is of great interest in view of the presence of "phytin" (inosite-hexaphosphoric acid) in the rapidly growing parts of plants[15] and in milk,[16] and its probable importance in connection with the growth of tissues.[16]

Physiological Actions of Tethelin, Other Than Its Action upon Growth.

The characteristic physiological phenomena following administration of the posterior lobe of the pituitary body, or extracts

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[14] Maquenne, M., *Ann. chim. et phys.*, 6th series, 1887, xii, 95.

[15] Posternak, S., *Compt. rend. Acad.*, 1903, cxxxvii, 202, 337, 439. Schulze, E., and Winterstein, E., *Z. physiol. Chem.*, 1896, xxii, 91; 1904, xl, 120. Neuberg, C., *Biochem. Z.*, 1908, ix, 551, 557. Anderson, R. J., *J. Biol. Chem.*, 1914, xviii, 441; 1915, xx, 493.

[16] Starkenstein, E., *Biochem. Z.*, 1911, xxx, 56.

thereof, consist in a pronounced rise in blood pressure,[17] slowing and strengthening of the heart beat, stimulation of involuntary muscles,[18] and pronounced diuresis.[19] These effects, as the above cited authors and others have shown, are not brought about by anterior lobe administrations.

The intravenous administration of tethelin does not cause any appreciable rise in blood pressure; on the contrary very little effect consequent upon the administration is to be observed. Very large doses (50 mg. per kilo) dissolved in physiological saline and injected intravenously in rabbits (marginal vein of the ear) lead to a slight and transient fall in blood pressure,[20] followed in a few minutes by a return to normal (Fig. 1) and a slight increase in the amplitude of the heart beat (see the early part of the tracing in Fig. 2 which is a continuation, after 2 minutes' interval, of the tracing in Fig. 1). Repeated administrations of this dose at intervals of a few minutes lead on each occasion to a transient fall of blood pressure, while the amplitude of the heart beat increases considerably (Fig. 2). The fall in blood pressure, even after the third administration, making a total dose of no less than 150 mg. per kilo, is not nearly so great as that produced by the inhalation of a whiff of amyl nitrite (see the end of the tracing in Fig. 2).

That the intravenous administration of tethelin does not cause diuresis is shown by the following experiment.

A male rabbit, 6 pounds in weight, was catheterized. The contents of the bladder measured 3.0 cc. The catheter was allowed to remain in the bladder, and after a lapse of half an hour the urine was collected for a period of 1 hour. The amount passed in this hour was 4.5 cc. 7.5 cc. of a 2.5 per cent solution of tethelin in physiological saline (188 mg.) were

[17] Oliver, G., and Schäfer, E. A., J. Physiol., 1895, xviii, 277. Howell, W. H., J. Exp. Med., 1898, iii, 215, 245. Schäfer, E. A., and Vincent, S., J. Physiol., 1899, xxv, 87.

[18] Dale, H. H., J. Physiol., 1906, xxxiv, 196. Bell, W. B., Brit. Med. J., 1909, ii, 1609.

[19] Magnus, R., and Schäfer, E. A., J. Physiol., 1901-02, xxvii, p. ix. Schäfer, E. A., and Herring, P. T., Phil. Tr. Roy. Soc., London, 1906, cxcix, 1.

[20] Hamburger, W. W., Am. J. Physiol., 1910, xxvi, 178, has previously drawn attention to the existence of an alcohol-soluble depressor substance in the anterior lobe.

FIG

5 cc.,

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Each

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nitrite

thereof, consist in a pronounced rise in blood pressure,[17] slowing and strengthening of the heart beat, stimulation of involuntary muscles,[18] and pronounced diuresis.[19] These effects, as the above cited authors and others have shown, are not brought about by anterior lobe administrations.

The intravenous administration of tethelin does not cause any appreciable rise in blood pressure; on the contrary very little effect consequent upon the administration is to be observed. Very large doses (50 mg. per kilo) dissolved in physiological saline and injected intravenously in rabbits (marginal vein of the ear) lead to a slight and transient fall in blood pressure,[20] followed in a few minutes by a return to normal (Fig. 1) and a slight increase in the amplitude of the heart beat (see the early part of the tracing in Fig. 2 which is a continuation, after 2 minutes' interval, of the tracing in Fig. 1). Repeated administrations of this dose at intervals of a few minutes lead on each occasion to a transient fall of blood pressure, while the amplitude of the heart beat increases considerably (Fig. 2). The fall in blood pressure, even after the third administration, making a total dose of no less than 150 mg. per kilo, is not nearly so great as that produced by the inhalation of a whiff of amyl nitrite (see the end of the tracing in Fig. 2).

That the intravenous administration of tethelin does not cause diuresis is shown by the following experiment.

A male rabbit, 6 pounds in weight, was catheterized. The contents of the bladder measured 3.0 cc. The catheter was allowed to remain in the bladder, and after a lapse of half an hour the urine was collected for a period of 1 hour. The amount passed in this hour was 4.5 cc. 7.5 cc. of a 2.5 per cent solution of tethelin in physiological saline (188 mg.) were

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[17] Oliver, G., and Schäfer, E. A., *J. Physiol.*, 1895, xviii, 277. Howell, W. H., *J. Exp. Med.*, 1898, iii, 215, 245. Schäfer, E. A., and Vincent, S., *J. Physiol.*, 1899, xxv, 87.

[18] Dale, H. H., *J. Physiol.*, 1906, xxxiv, 196. Bell, W. B., *Brit. Med. J.*, 1909, ii, 1609.

[19] Magnus, R., and Schäfer, E. A., *J. Physiol.*, 1901-02, xxvii, p. ix. Schäfer, E. A., and Herring, P. T., *Phil. Tr. Roy. Soc., London*, 1906, cxcix, 1.

[20] Hamburger, W. W., *Am. J. Physiol.*, 1910, xxvi, 178, has previously drawn attention to the existence of an alcohol-soluble depressor substance in the anterior lobe.

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now injected into the marginal vein of the ear. During the succeeding hour only 2.0 cc. of urine were passed, and during the hour following that only 1.5 cc. In order to ascertain whether the kidneys of this animal responded normally to diuretic agents, 5 cc. of a 1 per cent solution of barium chloride were now similarly administered. During the hour succeeding this, over 15 cc. of urine were passed.

It is clear, therefore, that at least in so far as blood pressure and diuresis effects are concerned, tethelin does not exert the physiological actions which are characteristic of posterior lobe preparations or extracts.

SUMMARY.

Tethelin, which has been shown in the preceding article to be the growth-controlling principle of the anterior lobe of the pituitary body, is soluble in water, ethyl alcohol, ethyl ether, chloroform, and carbon tetrachloride. It is insoluble in a mixture of one part by volume of absolute alcohol and one and one-half parts of dry ether. It contains 1.4 per cent of phosphorus, and nitrogen in the proportion of four atoms of nitrogen for every atom of phosphorus, two of the atoms of nitrogen being present in amino groups and one in an imino group which is converted into an amino group by hydrolysis with barium hydroxide. Among the products yielded by hydrolysis with barium hydroxide followed by hydrolysis with dilute sulfuric acid is found *dl*-inosite.

Tethelin probably contains an iminazolyl group, and to this extent may be regarded as being related to the physiologically active substances of the posterior lobe of the pituitary body. It does not, however, possess the characteristic physiological activity of these substances, relatively large doses administered intravenously to rabbits (50 mg. per kilo of body weight) producing only a very slight, transient fall in blood pressure and no diuresis.

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

Tenth Annual Meeting.

Boston, Mass., December 27–29, 1915.

PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

PRESIDENTIAL ADDRESS.

AN INDIRECT METHOD OF DETERMINING PYRIMIDINE GROUPS IN NUCLEOTIDES.

By WALTER JONES.

When the question of a presidential address was recently brought to my attention, I chose the title which appears in the program ("Methods in Biochemistry") and prepared to deal with the development of biochemistry in its broader aspects and with the vital rôle that methods have played in this development. But a number of considerations, especially the great length of the program, have induced me to abandon my original intention and confine the discussion to a particular chemical method which I have recently studied in collaboration with Miss Catherine Riley of Washington.

Questions pertaining to the chemistry of nucleic acid can be conveniently referred to the following formula:

$$
\begin{array}{l}
\text{HO} \\
\quad\diagdown \\
\text{O} = \text{P} - \text{O} . C_5H_8O_3 . C_5H_4N_5O \\
\quad\diagup \\
\text{O} \\
\quad\diagdown \\
\text{O} = \text{P} - \text{O} . C_5H_8O_3 . C_5H_4N_5 \\
\quad\diagup \\
\text{O} \\
\quad\diagdown \\
\text{O} = \text{P} - \text{O} . C_5H_8O_3 . C_4H_3N_2O_2 \\
\quad\diagup \\
\text{O} \\
\quad\diagdown \\
\text{O} = \text{P} - \text{O} . C_5H_8O_3 . C_4H_4N_3O \\
\text{HO}\diagup
\end{array}
$$
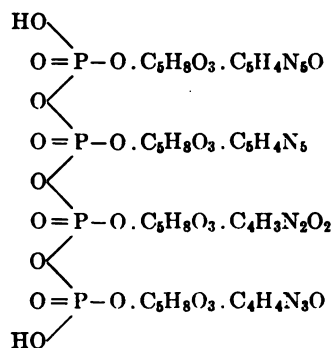
This formula represents nucleic acid as a tetra-nucleotide made up of four mono-nucleotides united to one another by their phosphoric acid groups, and it indicates the paths along which the substance may be expected to undergo decomposition whether

by chemical means or under the influence of physiological agents. Such a decomposition could produce a variety of substances: tri-nucleotides, di-nucleotides, mono-nucleotides, nucleosides, pyrimidine derivatives, purine derivatives, pentose, and phosphoric acid. So that, while the hydrolytic decomposition of nucleic acid is not a difficult matter to discuss, its experimental investigation is almost impossible; for of the decomposition products enumerated, only one (phosphoric acid) admits of exact quantitative determination. The chief difficulty lies in the estimation of the pyrimidine derivatives. But since the *purine derivatives* can be estimated with a fair degree of accuracy, the principal obstruction to investigation in this field would be removed if one could devise a precise experimental method of determining the ratio of the number of combined purine groups to the number of combined pyrimidine groups. From this point of view the following experiments are of interest.

Ten portions of commercial yeast nucleic acid from the same specimen were weighed into appropriate vessels and in one of these the total phosphoric acid was determined. The other nine portions were submitted to hydrolysis with 5 per cent sulfuric acid at 100° for various periods from ¼ hour to 7½ hours and after removal of the guanine with ammonia, the phosphoric acid was precipitated with magnesia mixture and weighed as magnesium ammonium phosphate.[1] The weight of magnesium ammonium phosphate obtained in each experiment was divided by the weight of nucleic acid from which it was produced and from the comparable results so obtained (Table I, Column 4) the upper curve in Fig. 1 was constructed. (The method of construction is so obvious as to require no explanation.)

This curve admits of an easy interpretation. Its rapid initial rise, sharp turn, and termination in a straight line slightly inclined to the axis, show clearly that in the acid hydrolysis of yeast nucleic acid, phosphoric acid is progressively liberated from two sources. From the one, the production is rapid and complete in less than 2 hours; from the other, it is much slower, continues beyond the time consumed in these experiments, and is so regular that its amount is definitely seen to be equivalent to 10 mg. of

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[1] The admissibility of magnesium ammonium phosphate as a form for weighing phosphoric acid will be taken up in a special contribution.

TABLE I.

Hydrolysis of Commercial Yeast Nucleic Acid with 5 Per Cent Sulfuric Acid 20 Cc. per Gm.

Nucleic acid.	Duration of hydrolysis.	Magnesium ammonium phosphate MgNH₄PO₄.6H₂O.			
		Obtained.	Per gm. of nucleic acid.	Pyrimidine correction.	From purine nucleotides.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
1.1782	¼	0.1060	0.090	0.003	0.087
0.7347	½	0.1284	0.175	0.005	0.170
0.8644	1	0.2315	0.269	0.010	0.259
0.9837	2	0.3125	0.318	0.020	0.298
0.9251	3	0.3038	0.329	0.030	0.299
1.0305	4	0.3488	0.338	0.040	0.298
0.8333	5	0.2876	0.345	0.050	0.295
0.9927	6	0.3565	0.359	0.060	0.299
0.9667	7½	0.3625	0.375	0.075	0.300
0.8179	Total.........0.5112		0.590*	Half...........0.295	

* See note at end of article.

magnesium ammonium phosphate per hour. If we subtract this amount from the figures of Column 4, Table I, we obtain the figures of Column 6, from which the lower curve of Fig. 1 has been constructed. This curve ends in a straight line, parallel to the axis, whose ordinate corresponds to half the total phosphoric acid obtainable from nucleic acid; or in other words, the
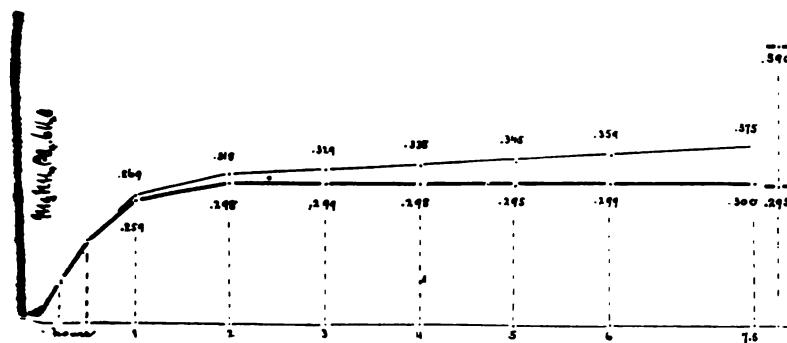
Fig. 1.

phosphoric acid which is rapidly and completely liberated by acid hydrolysis of yeast nucleic acid is half the total.

If time permitted it could easily be shown that at the point where the curve makes its sharp turn, the purines have been completely set free while the pyrimidines are still combined. Moreover the existing literature forces the conclusion that the two sources of phosphoric acid which we are discussing are the *purine* nucleotides and the *pyrimidine* nucleotides.[2] As the two sources of phosphoric acid are equal in amount the purine groups of nucleic acid must be equal in number to the pyrimidine groups.

TABLE II.

Purified Nucleic Acid.   First Fraction with Glacial Acetic Acid.

Nucleic acid.	Duration of hydrolysis.	Magnesium ammonium phosphate $MgNH_4PO_4.6H_2O$.			
		Obtained.	Per gm. of nucleic acid.	Pyrimidine correction.	From purine nucleotides.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
0.7519	1	0.1968	0.262	0.01	0.252
1.0308	2	0.3278	0.318	0.02	0.298
1.0024	3	0.3290	0.328	0.03	0.298
0.9703	4	0.3304	0.341	0.04	0.301
0.9009	5	0.3115	0.346	0.05	0.296
0.6846	Total.........0.4265		0.590*	Half..........0.295	

* See note at end of article.

This proposition has been accepted for a long time but rests only upon negative evidence, and its authorship would be difficult to discover if a third pyrimidine derivative were to be found among the hydrolytic products of nucleic acid. Such a discovery would now simply lead to the assumption of a third purine group in nucleic acid.

8 years ago Levene purified commercial yeast nucleic acid by dissolving the substance in ammonia and precipitating with glacial acetic acid.[3] This method of purification is of considerable importance because it was afterwards used by Levene and

[2] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1911, xliv, 1027.
[3] Levene, P. A., *Biochem. Z.*, 1909, xvii, 120.

his coworkers in their work on nucleotides, and it is naturally
of interest to know how such a preparation of nucleic acid would
conduct itself when submitted to the method of partial hydrolysis
described. Commercial yeast nucleic acid was therefore puri-
fied by Levene's method but was *fractionally* precipitated with
glacial acetic acid and in such a way that five preparations were
obtained. The 1st, 4th, and 5th of these were examined and the
results are shown in Tables II, III, and IV, and Figs. 2, 3, and 4.

As far as concerns the liberation of phosphoric acid by acid
hydrolysis, these three preparations differ neither from one another
nor from commercial nucleic acid. They contain different amounts
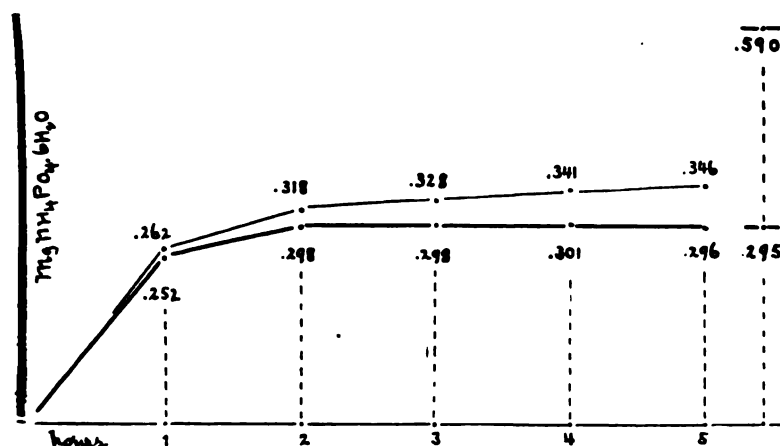
FIG. 2.

Of moisture and therefore give different quantities of phosphoric
acid, but each gives off accurately half of its phosphoric acid
rapidly and the other half slowly; each contains an equal number
of purine and pyrimidine groups.

To summarize: The purine-phosphoric acid of a nucleotide
preparation may be determined by heating the preparation for
2 or more hours with 5 per cent sulfuric acid and subtracting
from the estimated free phosphoric acid the equivalent of 10 mg.
of magnesium ammonium phosphate per hour. The pyrimidine
phosphoric acid is the difference between this value and the
total phosphoric acid.

TABLE III.

Purified Nucleic Acid. Fourth Fraction with Glacial Acetic Acid.

Nucleic acid.	Duration of hydrolysis.	Magnesium ammonium phosphate MgNH₄PO₄.6H₂O.			
		Obtained.	Per gm. of nucleic acid.	Pyrimidine correction.	From purine nucleotides.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
1.1669	1	0.3097	0.266	0.01	0.256
0.9881	2	0.3334	0.338	0.02	0.318
0.8772	3	0.3087	0.352	0.03	0.322
0.8238	4	0.2966	0.360	0.04	0.320
0.6984	5	0.2578	0.369	0.05	0.319
0.8350	Total.........0.5448		0.632*	Half..........0.316	

* See note at end of article.

The method thus briefly described was studied with yeast nucleic acid but was expected to be principally useful in its application to other nucleotides. Some of these applications have already been made and will form the subjects of future communications.

The preparations of nucleic acid employed in this work contain magnesium. Its amount is small and varies considerably from preparation to
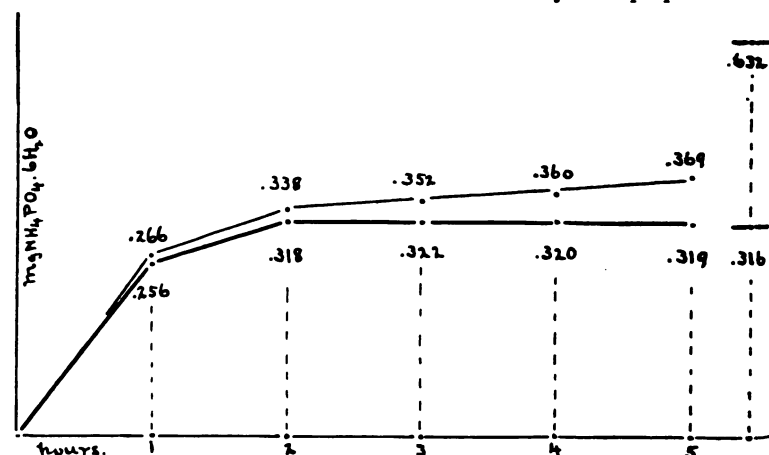
FIG. 3.

TABLE IV.

Purified Nucleic Acid. Fifth Fraction with Glacial Acetic Acid.

Nucleic acid.	Duration of hydrolysis.	Magnesium ammonium phosphate MgNH₄PO₄.6H₂O.			
		Obtained.	Per gm. of nucleic acid.	Pyrimidine correction.	From purine nucleotides.
gm.	*hrs.*	*gm.*	*gm.*	*gm.*	*gm.*
0.8805	1	0.2289	0.260	0.01	0.250
0.8434	2	0.2743	0.325	0.02	0.305
0.8532	3	0.2866	0.336	0.03	0.306
0.7346	4	0.2571	0.350	0.04	0.310
0.6624	5	0.2387	0.359	0.05	0.309
0.5307	Total.........0.3274		0.612*	Half.........0.306	

* See note at end of article.

Preparation so that it is not to be regarded as a constituent of the nucleic acid molecule. But its presence produces a slight disturbance in these experiments. When the products of hydrolysis are treated with ammonia for the removal of guanine some phosphoric acid is at the same time precipitated as magnesium ammonium phosphate and thus escapes estimation. In order to correct this error, the magnesium was determined in a large quantity of each preparation and its calculated equivalent of magnesium ammonium phosphate was subtracted from half that obtained in the total phosphoric acid determinations. The results are given in Table V.
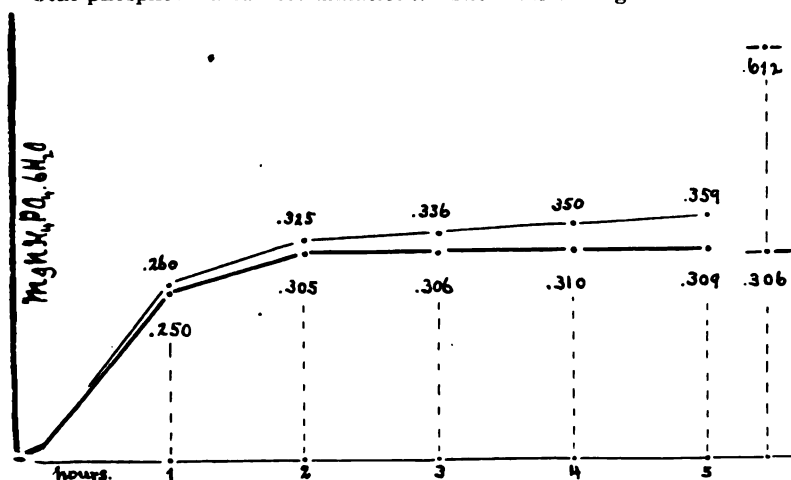
FIG. 4.

In this table have been included the results of moisture determinations and calculated total phosphorus percentages for the dry substances. The latter offer a curious suggestion.

It is not easy to determine the moisture of nucleic acid. A preparation can be brought to a fairly constant weight at any given temperature but will continue to lose weight when the temperature is raised. One is therefore somewhat at a loss to decide at what temperature the drying should be done. But in order to obtain as high phosphorus percentages as could

TABLE V.

The Phosphorus Percentages of Various Preparations of Nucleic Acid.

	Commercial yeast nucleic acid.	Nucleic acid fractioned with glacial acetic acid.					Levene's preparation.	Theoretical.
		1st fraction.	2nd fraction.	3rd fraction.	4th fraction.	5th fraction.		
MgNH₄PO₄. 6H₂O obtained per gm. of nucleic acid.	0.625	0.623	0.617	0.625	0.652	0.617		
Calculated half.	0.313	0.312	0.309	0.313	0.326	0.309		
Magnesium correction.	0.018	0.017	0.017	0.015	0.010	0.003		
Values found in preceding tables.	0.295	0.295	(0.292)	(0.298)	0.316	0.306		
Maximum moisture.	8.5	6.3	7.6	6.0	3.9	8.3		
Phosphorus percentage calculated for the dry substance.	8.67	8.45	8.48	8.44	8.61	8.55	8.60	9.52

reasonably be accepted, the preparations of nucleic acid were heated at 105° until the appearance of a pale yellow color showed that decomposition had begun. The total loss in weight was assumed to be moisture and was used as a basis for calculating the phosphorus of the dry preparations. High results were also secured by neglecting the magnesium correction in calculating the total phosphorus, although the magnesium impurity may well be magnesium phosphate. The phosphorus percentages agree closely with one another (8.67, 8.45, 8.48, 8.61, 8.55) and with the one reported by Levene[2] (8.60), but differ from that required by the formula for yeast nucleic acid (9.52).

ABSTRACTS OF SCIENTIFIC PROCEEDINGS.

ON FAT ASSIMILATION.

By W. R. BLOOR.

(From the Laboratory of Biochemistry, Harvard Medical School, Boston.)

In a previous communication[4] evidence has been presented to show that the lecithin of the whole blood increases during fat absorption. The present paper is·a report of further work along the same line in an attempt to locate the point of lecithin production. For this purpose dogs were given a feeding of olive oil and then blood samples were taken at once and every 2 hours after for 8 hours. Analyses were made for total fatty acids, cholesterol, and lecithin in both whole blood aňd plasma, from which, knowing the percentage of corpuscles in the blood, the content of the corpuscles in each of the above constituents was calculated. Eight experiments on two dogs were carried out. The results showed: (1) as regards total fatty acids, increases in both plasma and corpuscles, being greater in the latter and indicating that the corpuscles take up the absorbed fat from the plasma; (2) as regards cholesterol, no notable changes; (3) as regards lecithin, a great increase in the corpuscles with a slight increase in the plasma. The results are believed to show (1) that lecithin is the first step in the transformations of the fats in metabolism and (2) that the lecithin formation takes place in the corpuscles.

FAT INFILTRATION OF THE CAT'S KIDNEY.

By V. H. MOTTRAM.

The fatty acids of organs, such as the liver, heart, and kidney, are well known to be more unsaturated than those found in the fat in other parts of the body. And in cases investigated up to the present it is found that the iodine value of the fatty acids of the liver is almost invariably between that of the fatty acids of lipoid tissue and that of heart and kidney fatty acids. The only exception is that of the fatty acids of the cat's

[4] Bloor, W. R., *J. Biol. Chem.*, 1915, xxiii, 317.

kidney.[5] The subject has been reinvestigated as follows. Cats were kept in quiet surroundings on a constant liberal diet of oatmeal, milk, and meat for at least a month. They were then killed by bleeding under narcosis and estimations were made of percentage and iodine values of the fatty acids of the liver and right and left kidneys, and of the iodine value of the dépôt fatty acids. Histological preparations of the kidneys were also made.

The results of the experiments were (1) that the iodine values of the fatty acids of the kidneys never reached the level of the liver fatty acids; (2) that in some cases they were slightly above those of the fatty acids of lipoid tissue; (3) that in others they were strikingly below the latter, in which case the fatty acids were fluid at room temperature; (4) that the iodine values of the fatty acids were inversely proportional to the percentage present, suggesting an infiltration with milk fatty acids (iodine value 20 to 30); and (5) that this interpretation was borne out by histological preparations. In the latter, where there was much fat present, it was invariably in the convoluted tubules and rarely in the straight tubules; in some cases fat could be made out in the blood vessels (lipemia) and in others in the lumina of the convoluted tubules (lipuria?). Nuclear chromatin was clear and stained sharply.

Conclusions to be drawn from this research are that (1) desaturation of cat's kidney fatty acids much beyond that of the fatty acids of lipoid tissue is not normal (contrast with man, dog, goat, pig, rabbit); i.e., if cat's kidneys metabolize fatty acid as a source of chemical energy they can utilize normal fatty acids; and (2) in the presence of fat in the food and blood stream, cat's kidneys show a marked infiltration with fat.

THE COMPOSITION OF ADIPOCERE.

By R. F. RUTTAN and M. J. MARSHALL.

.The tabulated results of the quantitative analysis are as follows:

	per cent
Palmitic acid	67.52
Stearic acid	3.3

[5] Leathes, J. B., Lancet, 1909, i, 95.

	per cent
Oleic acid	5.24
ι-Hydroxy stearic acid	9.48
κ-Hydroxy stearic acid	6.32
Stearin and palmitin	1.21
Olein	0.16
Unsaponified matter	0.87
Calcium soaps	4.41
Protein	0.665
Ash	0.578
Humus and undetermined	0.247
	100.000

The fatty acids and traces of fats, etc., soluble in ether consti-tuted 94.1 per cent of the adipocere and gave the following physical and chemical constants:

Specific gravity at 100°C	0.8436
Refractive index at 60°	1.436
Melting point	60–63°C.
Acid value	201.7
Saponification value	207.0
Iodine value	6.04
Acetyl value	34.75

The total nitrogen present was only 0.1778 per cent, of which only 0.0289 per cent was ammonia nitrogen.

As a result of the studies recorded in this paper we are justified in concluding that adipocere is the residue of the preexisting fats of animals. It is composed almost entirely of the insoluble fatty acids left after the slow hydrolysis of the fats in wet ground. The protein matter has entirely disappeared and the glycerol, soaps, etc., resulting from the hydrolysis, are carried away in aqueous solution.

The insoluble hydroxy stearic acids which are so characteristic of adipocere are derived from a portion of the oleic acid in the original fat by hydration.

No margaric acid or hydroxy margaric acid is present. Ammonium and other soluble soaps are absent and only the calcium salts are present in small quantity in a matured specimen of adipocere such as that reported upon.

ON THE RÔLE PLAYED BY ELECTROLYTES IN DETERMIN THE PERMEABILITY OF PROTOPLASM.

By G. H. A. CLOWES.

(*From the Biological-Chemical Laboratory of the State Institute for the , of Malignant Disease, Buffalo.*)

If aqueous solutions of soap or caustic soda are prep sufficiently concentrated to give a system of fifty drops passed through olive oil from a Traube stalagmometer, an: crease in the amount of NaOH or soap causes a consider increase in the number of drops, and any decrease causes a re tion. Similarly salts of monovalent cations, Li, Na, K, cause an increase in the number of drops, while salts of div and trivalent cations, Ca, Sr, Ba, Fe, Al, etc., cause a reduc Furthermore, when salts of monovalent cations are employe conjunction with salts of divalent cations, they are found to ance one another or exert a compensatory effect upon one anc in certain ratios, which are those most commonly observe studying the antagonistic effects of various salts upon one anc in biological systems. NaCl, for example, is balanced by mea $CaCl_2$ in molecular ratios varying from one to two of the latt 100 of the former, according to concentration. While alks and tri- and divalent anions cause an increase in the numb drops, acids, up to a certain concentration, and tri- and div cations cause a reduction in the number of drops. Since a film is formed at points of contact between oil and water, number of drops formed is an index of the resistance of the in question, or the readiness with which it is dispersed or pep by the constituents of the aqueous phase.

It appears, therefore, that electrolytes may be divided re. into two classes; those which promote dispersion of the soap and consequently increase its permeability, and those which mote aggregation of the soap film and consequently diminis permeability. It is interesting to note that these subst. exert effects both qualitatively and quantitatively on soap which correspond with those observed by Loeb, Lillie, Osterl and others, while studying the antagonistic effects exerte electrolytes on living cells. Salts of Mg and anesthetics, employed in suitably constituted systems, exert at certain

mum points a maximum protective effect upon the film, but when employed at higher concentrations exert a destructive effect upon the film, and the concentrations at which these effects are produced correspond very closely with those recorded by Lillie and others in studying the protective effect exerted by anesthetics on marine organisms.

In upwards of 100 experiments parallel results have been obtained in physical and biological systems, even to the extent of observing substances like Mg functioning in one case as an antagonist to calcium in ratios of 2 : 1, and in another case exerting the same effect as calcium when functioning as an antagonist to NaCl in ratios of one or two to 100. On the basis of these experiments a theory has been developed regarding the equilibrium of protoplasmic systems, further details regarding which will be published shortly, based on the assumption that adsorbed ions, by influencing the surface tension of concentration films promote or retard the dispersion of these films in the external aqueous medium surrounding protoplasm, and thus promote or retard the intermittent intercommunication between the interior aqueous phase of protoplasm and the exterior aqueous medium, which may be assumed to take place normally under the influence of varying concentrations of OH resulting from varying concentrations of CO_2 and other substances produced in the course of normal metabolism.

A COMPARISON OF THE SÖRENSEN, VAN SLYKE, AND COLORIMETRIC METHODS FOR THE ESTIMATION OF PROTEIN HYDROLYSIS.

By VICTOR JOHN HARDING AND REGINALD M. MacLEAN.

A study was made of the hydrolysis of various proteins by pancreatic extracts, and the proteoclasis was followed by the Sörensen formaldehyde titration method, the Van Slyke gasometric method, and the authors' new colorimetric method. The last depends upon the quantitative application of the ninhydrin reaction. The representative proteins used were: serum albumin and globulin, gluten, fibrin, gelatin, casein, nucleoprotein, and two samples of peptone.

The results uniformly showed that the Van Slyke and the authors' methods gave identical results which were higher than those given by the Sörensen method. A discrepancy between the results of the colorimetric and gasometric methods was sometimes observed, however, when applied to native proteins, the colorimetric method giving lower figures. This was believed to be due to the reactivity of the ninhydrin reaction being confined chiefly to α-amino groups.

THE DISTRIBUTION IN FOODS OF THE SO CALLED VITAMINES AND THEIR ISOLATION.

By M. X. SULLIVAN and CARL VOEGTLIN.

(From the Pellagra Hospital, United States Public Health Service, Spartanburg, S. C.)

Antineuritic substance or substances were found in wheat bran, in the common black-eyed cow-pea of the Southern States, and in ox liver, but not in pork fat. In isolating the vitamine fraction, various modifications of Funk's methods were tried. The most satisfactory method is as follows: The hot alcohol-soluble residue was hydrolyzed 5 hours at 90°C. with 5 or 10 per cent H_2SO_4 in a current of CO_2. The cold filtrate was precipitated by phosphotungstic acid. The resulting precipitate was decomposed either by rubbing with neutral lead acetate, or by dissolving the precipitate in 50 per cent alcohol and treating the solution with excess of lead acetate. The filtrate from the lead precipitate was freed from Pb by H_2S and concentrated in vacuo at 50°C. The solution filtered was precipitated with considerable excess of silver acetate. The filtrate was made slightly alkaline with $Ba(OH)_2$. The resulting precipitate, suspended in water and made slightly acid with H_2SO_4, was decomposed by H_2S. The AgS filtrate was concentrated in vacuo to a small volume and brought to a slight acidity by means of dilute Na_2CO_3. The method here given avoids the possible destructive action of $Ba(OH)_2$ in the decomposition of the phosphotungstic precipitate and the oxidizing action of silver nitrate in the precipitation of the vitamine fraction.

By treating the alcohol residue of liver and peas with water and extracting with ether an aqueous solution can be obtained

free from lecithin. This aqueous solution on hydrolysis yielded a crude antineuritic preparation which was found non-toxic in moderate doses.

Chickens fed on corn-bread made from corn-meal, milk, and baking soda (sodium bicarbonate) rapidly developed polyneuritis, while those fed on corn-bread made from corn-meal, milk, and sodium chloride remained in good condition.

THE RELATION OF LIPOIDS TO VITAMINES.

By M. X. SULLIVAN and CARL VOEGTLIN.

(From the Pellagra Hospital, United States Public Health Service, Spartanburg, S. C.)

On extracting wheat bran with 90 to 95 per cent alcohol and removing the alcohol *in vacuo* at 50°C. a brownish yellow lipoid mass is obtained. This lipoid mass is soluble in chloroform and ether but has little solubility in cold water. The crude material possesses little, if any, curative power when given to fowl with polyneuritis, but after hydrolysis with acid and proper fractionation a vitamine fraction possessing strong antineuritic properties is obtained.

In the presence of considerable water, however, ether extraction of the lipoid mass yielded three layers, an ethereal layer, a middle semisolid layer, and an aqueous layer.

When these layers were separately hydrolyzed and fractionated to the vitamine fraction only the aqueous layer yielded a fraction possessing antineuritic properties.

It is obvious that if vitamine-containing foodstuffs rich in fat and lipoids and practically free from water be extracted with lipoid solvents, such as chloroform or ether, the erroneous conclusion might be drawn that the antineuritic substance or substances are constituents of lipoids *per se*.

In reality the antineuritic material is either dissolved in, or mixed in the lipoid or is in easily separable combination with it.

A SIMPLIFIED PROCEDURE FOR THE DETERMINATION OF THE CARBON DIOXIDE TENSION IN. THE ALVEOLAR AIR.

By W. McK. MARRIOTT.

(From the Department of Pediatrics, Johns Hopkins University.)

A modification of the Plesch method for the collection of air samples is described. A simply constructed mask renders the collection possible with infants and with laboratory animals.

The carbon dioxide tension in the sample is determined by bubbling the air through a 0.01 N solution of sodium bicarbonate containing phenolsulphonephthalein as an indicator. The reaction of the solution and the corresponding color obtained are functions of the carbon dioxide tension. The color is compared with that of standard solutions of mixed phosphates containing the indicator, made up in such a way that the tension of carbon dioxide in mm. may be directly read off. These standard solutions are practically permanent. Slight changes in temperature are without effect on the accuracy of the method. The temperature, however, may be easily controlled by immersing the tubes in a beaker of water at the temperature selected as a normal (20°C.) Changes in the barometric pressure are, from the nature of the method, entirely without influence.

Results invariably agree closely with those obtained by the analysis of the air sample in the usual Haldane gas analysis apparatus.

THE USE OF A NEW REAGENT FOR MICROCOLORIMETRIC ANALYSIS AS APPLIED TO THE DETERMINATION OF CALCIUM AND OF INORGANIC PHOSPHATES IN THE BLOOD SERUM.

By JOHN HOWLAND, F. H. HAESSLER, and W. McK. MARRIOTT.

(From the Department of Pediatrics, Johns Hopkins University.)

The methods described are based on the fact that the red color of a solution of ferric thiocyanate is discharged by certain substances, among which are oxalates and phosphates.

Calcium is precipitated as the oxalate, dissolved in acid, added to a standard solution of ferric thiocyanate, and made up to a definite volume. The color of the resulting solution is compared

with that of a solution containing known amounts of calcium oxalate and ferric thiocyanate. The phosphates are precipitated as a magnesium and ammonium phosphate. The precipitate is dissolved and color comparisons are made as above.

The calcium content of the serum in children was found to be normal or but slightly diminished in most cases of rickets studied. When the rickets was accompanied by evidences of tetany the calcium content of the serum was very low. In dogs there was found a progressive diminution in the calcium of the serum, following parathyroidectomy.

Normal human serum contains very small amounts of inorganic phosphates. The amount, however, has been found to be markedly increased in certain conditions in infants accompanied by acidosis and characterized by diminished renal function. In these cases acid phosphate is apparently retained through failure of the excretory activity of the kidney.

The principle utilized in the methods described is applicable to the determination of other substances, especially lactic acid, and is at present being used for that purpose.

CONTINUOUS INTRAVENOUS INJECTIONS AT UNIFORM RATES.

By W. D. SANSUM, RUSSEL M. WILDER, and R. T. WOODYATT.

(From the Otho S. A. Sprague Memorial Institute Laboratory for Clinical Research, Rush Medical College, Chicago.)

A demonstration is given of a motor-driven quantitative pump for continuous intravascular administrations, perfusions, etc., at controllable rates, with a discussion of its application in the study of quantitative aspects of absorption, metabolism, and secretion, and details of experiments in which the method was applied in the study of glucose, galactose, levulose, lactose, and glyceric aldehyde.

The intravenous tolerance limit expressed as a velocity has been found for glucose at close to 0.85 gm. per kg. of body weight hourly, for levulose close to 0.15 gm., for galactose and glyceric aldehyde close to 0.1 gm., for lactose close to 0.0 gm. per kg. per hour. Glucose may be given by vein to normal resting rabbits, dogs, or men at rates below 0.85 gm. per kg. per hour for 2 to 12 hours, with no glycosuria and no acceleration of diuresis.

At rates of injection above this limit, glycosuria and diuresis appear. The rate of glycosuria becomes constant at a level characteristic for each uniform injection rate, provided all experimental conditions are appropriate. It may require 4 to 5 hours for the excretions to become constant with higher injection rates. Observations were presented on sugar-, salt-, and fever-diuresis and water balance.

THE PRODUCTION OF HYPERGLYCEMIA AND GLYCOSURIA BY MAGNESIUM SALTS.

By I. S. KLEINER and S. J. MELTZER.

(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)

In their experiments on the action of magnesium salts, Meltzer and Auer[6] observed that after subcutaneous injections of magnesium sulfate the urine of rabbits contains a reducing substance. Underhill and Closson,[7] who later noticed the presence of hyperglycemia after an intravenous injection of magnesium sulfate, ascribed the hyperglycemia to the asphyxia which the magnesium salts produced in their experiment.

In a series of experiments which we have recently carried out on dogs, all the animals had from the beginning to the end of the experiment either intratracheal insufflation or the usual artificial respiration. The occurrence of asphyxia was thus excluded. The operative part was done under local anesthesia. In most of the experiments a $\frac{M}{4}$ solution of magnesium sulfate was injected intravenously. There was a considerable increase of the sugar content of the blood after infusion in practically all experiments. In most cases the original glycemia did not exceed 0.13 per cent, while at the end of the injection or some time later, the sugar content of the blood was often as high as 0.4 per cent and even higher. In blood taken about $1\frac{1}{2}$ hours after the end of an injection the glycemia was often found to have dropped to the original content.

There can be no doubt that magnesium sulfate produces considerable hyperglycemia which is not due to asphyxia; it is

[6] Meltzer, S. J., and Auer, J., Am. J. Physiol., 1905, xiv, 371.

[7] Underhill, F. P., and Closson, O. E., Am. J. Physiol., 1906, xv, 321.

produced in some way specifically by the magnesium salt. (In an experiment with sodium sulfate no hyperglycemia was produced.)

It is a noteworthy fact that the glycosuria was very little marked and not in proportion to the hyperglycemia. Glycosuria was often entirely absent and when present it never reached even 0.5 per cent. The intravenous injection of magnesium sulfate also produced very little diuresis.

EXPERIMENTAL AND CLINICAL STUDIES ON MENTAL DEFECTIVES.

II. THE GLYCOSURIC REACTION OF INSTITUTION INMATES IN RELATION TO NUTRITIONAL AND PATHOLOGICAL CONDITIONS.

By AMOS W. PETERS.

With the Assistance of Mary E. Turnbull and Caroline D. Blackburn.

(From the Biochemical Laboratory, Training School, Vineland, N. J.)

Feeble-minded inmates of an institution were tested for dextrose tolerance with the result that the nutritional factor had a more general significance than the pathological. The method permitted the estimation of 0.01 per cent of sugar in clarified urine. The degree of tolerance was expressed as:

$$\text{Saturation capacity} = \frac{\text{Sugar ingested, gm.}}{\text{Body weight, kg.} \times \text{sugar excreted, gm.}}$$

The number of units obtained shows the capacity of the organism to absorb sugar before the renal overflow occurs.

The age group of 5 to 12 years, of both sexes, showed a high saturation capacity of 269 units compared to 96 units for the age group 13 to 37 years, with low tolerance of 61 units for females in the latter group. Repetitions showed a high degree of constancy. There was a low tolerance group of 49 units, mostly of "disturbed" females, and a Mongolian group showing high tolerance of 205 units. The only pathological groups found were the low tolerances and the Mongolians, excessively high toler-

ance being the rule with 72 per cent of the cases. The notably high dosages required for the cases as a whole were due to chronic undernutrition and defective institutional diet.

THE DETERMINATION OF CALCIUM IN BLOOD.

By JOHN O. HALVERSON and OLAF BERGEIM.

(From the Department of Physiological Chemistry of Jefferson Medical College, Philadelphia.)

A method was suggested for the determination of calcium in whole blood, plasma, or serum. Ordinarily about 10 cc. of whole blood were used. It was deproteinized by means of picric acid in strongly acid solution and the calcium precipitated as oxalate directly in the filtrate according to McCrudden's procedure. The precipitate was washed by centrifugation and the oxalate titrated with 0.01 N permanganate solution under standardized conditions.

Magnesium was determined in the filtrate after destruction of ammonium salts. The precipitation as magnesium ammonium phosphate and the washing of this precipitate were carried out in centrifuge tubes. The ammonia content of the precipitate was determined colorimetrically after distillation or by titration using methyl red as an indicator. This was found preferable to the nephelometric determination of the phosphorus content except where less than 2 cc. of blood were available.

A COMPLETE METABOLISM STUDY OF GOITER WITH THE EFFECT OF THYROID AND THYMUS TREATMENT.

By JOHN O. HALVERSON, OLAF BERGEIM, and PHILIP B. HAWK.

(From the Department of Physiological Chemistry of Jefferson Medical College, Philadelphia.)

The metabolism of nitrogen, phosphorus, sulfur, calcium, and magnesium was studied in a case of exophthalmic goiter which appeared to respond readily to thyroid and thymus treatment.

The study was made in five periods of 5 days each: (1) a control period; (2) a period with thyroid treatment; (3) a control period; (4) a period with thymus treatment; (5) a control period. The patient was on a constant food and water intake throughout.

In Period 1 there was a marked loss of nitrogen, phosphorus, and magnesium, while the sulfur and calcium were practically balanced. On thyroid treatment marked retentions were observed for all the elements, the retention reaching a maximum for nitrogen, sulfur, and phosphorus in the final period and for calcium and magnesium in the third period. These retentions continued throughout the experiment and indicate a stimulation of metabolism by thyroid. Thymus treatment, however, appeared to depress this stimulating effect as higher retentions were observed in the post-thymus than in the thymus period. Thyroid treatment led to diuresis while on thymus treatment the urine volumes decreased greatly. Likewise the body weight decreased considerably on thyroid treatment and rose again on thymus administration. The fluctuations in weight could be accounted for on the basis of water loss and nitrogen elimination.

ORIGIN AND DETERMINATION OF CREATINE IN MUSCLE.

By L. BAUMANN, H. HINES, and J. MARKER.

(From the Laboratories of Internal Medicine and Physiology, Iowa State University, Iowa City.)

The figures obtained for creatine in muscle by the Janney and Blatherwick extraction method agree closely with those obtained by the acid hydrolysis method reported by Baumann two years ago. The older method has been simplified so that a complete determination may be carried out in less than half an hour after the extract is obtained. It may also be used to advantage in the determination of creatine in organs. All the substance in the hydrolyzed muscle extract responsible for the Jaffé reaction may be precipitated by picric acid and potassium.

Arginine, betaine, methylureidoacetic acid, sarcosine plus urea, and choline plus urea have been perfused through muscle tissue. Only choline plus urea consistently lead to an increase in muscle creatine.

IS THE GLUCOSE RETAINED, WHEN SODIUM CARBONATE IS ADMINISTERED TO DEPANCREATIZED DOGS, STORED AS GLYCOGEN?

By B. KRAMER and J. MARKER.

(*From the Physiological Laboratory of the State University of Iowa, Iowa City.*)

The experiments here reported are a few of a series covering a period of several years in which the object was to determine what happens to the glucose retained when sodium carbonate is administered to depancreatized dogs. The well known fact that in normal animals, both cold- and warm-blooded, sodium carbonate inhibits glycogenolysis, made the assumption plausible that the ability of sodium carbonate to lower the glycosuria and the D: N ratio in diabetic dogs might be attributed to some effect upon the glycogenetic or glycogenolytic mechanism.

Two distinct methods of procedure were adopted. First, a totally depancreatized dog was fed on meat and sodium carbonate until the urinary analysis showed that a considerable quantity of glucose had been retained. The animal was then quickly anesthetized, specimens of liver and muscle were removed, and the glycogen content was determined, and only traces were found. To corroborate this result, a similar diabetic animal was rendered glycogen-free by repeated injections of adrenalin. Then meat and carbonate were fed until considerable glucose, which must have come from protein, had been retained. The adrenalin administration was then resumed, and the failure to demonstrate extra sugar in the urine was interpreted as indicating that none of the glucose retained had been stored as glycogen. The saliva was frequently tested for glucose, and only minute traces were found. Gastric and intestinal washings failed to show a sufficient amount of reducing substances to account for more than a very small fraction of the glucose retained even when the sugar content of the blood must have been very high, as a result of combined effects of ether anesthesia, the operation, and the intravenous injection of a solution of glucose.

Conclusions.—Sugar retained when sodium carbonate is administered to depancreatized dogs is not stored as glycogen nor is it eliminated by way of the saliva or the gastro-intestinal tract.

THE INFLUENCE OF ALKALI ON THE DIABETES OF PARTIALLY AND TOTALLY DEPANCREATIZED DOGS.

By J. R. MURLIN and B. KRAMER.

(*From the Physiological Laboratory of the Cornell University Medical College, New York City.*)

Continuing the observations, reported to this society one year ago, on the influence of sodium carbonate on the glycosuria, the hyperglycemia, and the respiratory metabolism of depancreatized. dogs, we have to report today confirmation of the statements then made in the following particulars: (1) disappearance of sugar from the urine of a totally depancreatized animal within 4 hours following the infusion of 300 cc. of Ringer's solution containing 1 per cent Na_2CO_3; (2) no increase in the blood sugar accompanying this; (3) a rise of the respiratory quotient to 0.90, 0.85, and 0.85 in successive hours following the administration of Na_2CO_3 with glucose intravenously to a dog whose urine had been rendered sugar-free even while on full diet by the administration of sodium carbonate.

Of new observations we have the following to report: (1) no rise in R. Q. after the administration of Na_2CO_3 to normal dogs; (2) a limited capacity to oxidize glucose on the part of a dog in which a sufficient amount of pancreas had been left (at the main duct) to prevent glycosuria on a meat diet; (3) an increase in this capacity, as judged by the rise in R. Q. and an increase in heat production, when Na_2CO_3 was given with glucose, but not when Na_2CO_3 was given alone; (4) a marked rise in R. Q. when NaOH, in amount to make 0.1 N, added to Ringer's solution was administered intravenously to a totally diabetic dog on the 5th day following pancreatectomy.

The complete reports will appear in an early number of this *Journal.*

SOME TESTS OF THE DIGESTIBILITY OF KAFIR-CORN AND INDIAN CORN-MEAL PREPARED FOR THE TABLE IN THE USUAL WAY.

By C. F. LANGWORTHY and A. D. HOLMES.

(*From the Office of Home Economics, States Relations Service, United States Department of Agriculture.*)

In certain areas of the United States, too dry for the most successful cultivation of the better known food grains, the grain sorghums will flourish. The result is that much study has been made of the cultivation and possible uses of Kafir-corn, feterita, milo-maize, and other more or less similar crops. They are now well recognized foodstuffs in some other countries and have at least a limited use in the United States.

From Kafir-corn a meal is made which has many uses. To some palates, at least, it resembles buckwheat in flavor. Like corn-meal, it is not suitable alone for yeast-raised bread, owing to a lack of gluten, but can be used for making many other types of bread and similar foods familiar to every housewife who uses corn-meal. In view of these facts, it was of interest to the Department of Agriculture to include the grain sorghums in its studies of the food value and possible uses of agricultural products.

As the result of seven experiments with healthy young men as subjects, it appeared that Kafir-corn bread, when eaten as a part of a simple mixed diet, had, on an average, a digestibility of 46 per cent for protein and 97 per cent for carbohydrates. In other tests, under somewhat different conditions, the values for the digestibility of protein were a little lower.

For comparison, tests were made, under like experimental conditions, with corn-meal ground to the same degree of fineness as the Kafir-corn. The coefficients of digestibility of corn bread as shown by the average of three tests, were: protein, 55 per cent; carbohydrates, 97 per cent.

The composition and thoroughness of digestion, as well as the economic considerations involved, indicate that such grains as Kafir-corn are worthy of serious consideration as foodstuffs.

The experiments briefly cited form a small part of an extended study of grain sorghums and their uses, the results of which await publication.

THE DETERMINATION OF β-HYDROXYBUTYRIC ACID.

By P. A. SHAFFER and R. S. HUBBARD.

(From the Laboratory of Biological Chemistry, Washington University, St. Louis.)

A further revision of the conditions underlying the oxidation of β-hydroxybutyric acid by potassium bichromate and sulfuric acid has shown that the oxidation proceeds faster with increasing concentration of sulfuric acid, and at a concentration of about 10 N the oxidation may be completed in about 20 minutes. If the bichromate is added in dilute solution only as fast as it is reduced the yield of acetone is about 90 per cent of the amount theoretically formed from the hydroxybutyric acid present. Crotonic acid is not formed until the concentration of sulfuric acid reaches about 12.5 N. Based on these facts the procedure for the determination of hydroxybutyric acid may be greatly shortened in time without sacrifice in accuracy. Further modifications were given for the determination of acetone bodies in urine, blood, and tissues.

The authors were unable to confirm the claim of Folin and Denis that the oxidation of hydroxybutyric acid by the modified procedure described by them yields theoretical amounts of acetone; about 85 to 90 per cent is the maximum amount obtained under their conditions.

HYDROGEN ION CONCENTRATION IN AUTOLYSIS.[*]

By MAX MORSE.

Thymus was used for the experiments. Digests *in vitro* were conducted and the concentration of hydrogen ions was estimated at regular intervals for a period of 10 days by means of the Sörensen method for colorimetrically determining P_H. The initial determination gave $P_H = 6.4$. At the time when the curve of rate of autolysis as measured by the non-precipitable nitrogen reached its approximate maximum, the curve of P_H had done likewise ($P_H = 4.2$). Throughout, the two curves ran similarly, so that the conclusion seems justified that we are deal-

[*] Read by title.

ing with an autocatalytic phenomenon, wherein the acidity, due to lactic acid, phosphate, etc., acts as a true co-enzyme or catalyzer to accelerate the process, or there may be a change of physical state, such as Jobling and others have postulated for the enzyme actions studied by them. There is no evidence that there is any modification of substrate, on the part of the acid, rendering certain protein components digestible for the tissue enzyme.

CREATINE IN ATROPHY.[8]

By MAX MORSE.

Creatine estimations have been made on a number of children with various diseases involving atrophy, such as poliomyelitis, marasmus, recurrent vomiting, nephritis, lobar pneumonia, etc. Retention of creatinine has been observed in all nephritic cases and also in those of recurrent vomiting, wherein virtually a starvation condition results. One case of marasmus was followed through its entire range and it was clear that one could follow the patient's condition by the curve of creatinine-creatine ratio. In one case, turpentine nephritis, no creatinine could be determined in the urine for several days by any method used. In all cases without exception, creatine ran high, especially in the instances of anterior poliomyelitis, where a noticeable amount was excreted daily. Practically no work has been done upon the problem of correlating creatinine and creatine changes with pathologic states, and the value of such studies, theoretically and clinically, has been demonstrated by these studies.

The studies have been conducted in common with Dean Cutter of the University of Nebraska College of Medicine and the Child Saving Institute, Omaha.

GROWTH ON STRICTLY VEGETABLE DIETS.[9]

By E. B. HART and E. V. McCOLLUM.

We have published data from this laboratory showing that omnivora will not grow normally on certain grains or mixtures of grains unless there are added distinct supplements such as salts, proteins, accessories, or combinations of these. A number of

investigations by other workers have also been reported in the literature, supporting the view that strictly vegetable diets will not be sufficient for the growth and normal physiological condition of the organism. In fact the view that a strictly vegetable diet is insufficient has been emphasized from time to time by students of human nutrition. In contrast to this view are the obvious results of the complete development of herbivora on strictly vegetable diets. Guinea pigs, sheep, cattle, etc., often make 80 per cent of their growth from such sources.

It appeared probable, therefore, that the roughage or that part of the plant other than the seed was the material capable of supporting growth when fed alone or acting as a supplement to grains and grain mixtures. Failure of early experiments to maintain growth with grains and small daily allowances of green vegetable tissue is to be explained on the basis of an insufficiency in quantity of the stem or leafy part of the plant in the diet. The herbivora consume daily large masses of these materials.

In our own experiments, omnivora (swine and rats) maintained sustained growth and apparent physiological vigor when the grains were intimately mixed with a roughage so as to insure a liberal consumption of the latter. With 15 or 25 per cent of alfalfa meal in grain rations we have succeeded in maintaining growth and well-being in swine, whereas without this roughage complete failure results. The probable factors contributed by the roughage will be discussed in a future paper presenting these data.

SOME DIASTASE ACCELERATORS.

A PRELIMINARY REPORT.

By E. W. ROCKWOOD.

(From the Chemical Laboratory of the University of Iowa, Iowa City.)

The action of amino compounds was tested on the salivary and pancreatic amylases. There was a marked increase in the formation of reducing sugars in the presence of glycine, hippuric acid, and acetamide. Anthranilic acid and urea had no effect. The effect of the amino compounds is most evident when very small quantities of the ferments are used. The work is being continued.

UREASE CONTENT OF CERTAIN BEANS, WITH SPECIAL REFERENCE TO THE JACK BEAN.

By J. G. MATEER and E. K. MARSHALL, Jr.

(From the Laboratory of Pharmacology, Johns Hopkins University, Baltimore.)

A number of beans have been examined for urease. *Phaseolus calcaratus, Phaseolus angularis, Phaseolus aconitifolius, Phaseolus Mungo,* early Florida velvet bean, Chinese velvet bean, *Vigna sinensis, Dolichos Lablab, Stizolabium deeringianum,* do not contain the enzyme, whereas in *Phaseolus aureus, Lupinus albus, Dolichos biflorus,* sword bean, and jack bean it is present.

There exists great variability in the urease content of beans. The jack bean contains about fifteen times, and the sword bean about five times as much urease as the soy bean, while *Dolichos biflorus* contains about $\frac{1}{5}$, the white lupine about $\frac{1}{50}$, and *Phaseolus aureus* about $\frac{1}{350}$ as much as the soy bean. The aqueous extract of the jack bean does not decompose ereptones, casein, hippuric acid, leucyl-glycine, uric acid, creatinine, creatine, adenine. The jack bean aqueous extract contains about 25 mg. of solid material, while the aqueous extract of the soy bean contains about 40 mg. per 1 cc.

CONCERNING PROTEIN SYNTHESIS AND METABOLIC DISEASES

By N. W. JANNEY.

(From the Chemical Laboratory of the Montefiore Home and Hospital for Chronic Invalids, New York.)

There exists considerable indirect evidence that carbohydrates contribute material which enters into the synthesis of protein. The sparing action of glucose on protein, which cannot be adequately accounted for on dynamic grounds, may be due to protein repair and synthesis from intermediary products of glucose metabolism.

The glands of internal secretion promote growth, *i.e.,* the formation of new protoplasm, and are indispensable for the maintenance of its nutritional processes. These organs also regulate the distribution of carbohydrates in the organism. In view of the possible synthetic function of glucose, the ductless glands may

therefore function to regulate the supply of material for the synthetic repair, regeneration, and formation of living protein. Recent experiments by the writer can be adduced to support this hypothesis. It was shown that the sparing action of glucose on protein was increased by the additional feeding of minute doses of thyroid tissue.

If the synthesis of protein from carbohydrates should prove to take place extensively a change of view-point in regard to various metabolic problems might be necessary. The abnormalities of protein metabolism in thyroid disease accompanied by disturbances of carbohydrate metabolism, could be reasonably explained. The same holds true for many phenomena of diabetes. Aside from the glycosuria and accompanying symptoms, this disease is characterized by loss of protein to the organism, degenerative lesions and trophic symptoms indicating a disturbance of protoplasmic nutrition. These anomalies in diabetic metabolism may be due to an inadequate supply of synthetic intermediate substances normally produced from carbohydrates.

ENZYMES OF A CELLULOSE-DESTROYING FUNGUS FROM THE SOIL, PENICILLIUM PINOPHILUM.[1]

By E. D. CLARK and F. M. SCALES.

(*From the Bureau of Chemistry, United States Department of Agriculture, Washington.*)

The bacterial and fungus flora of soils is active in causing decomposition in both plant and animal matter. Scales[2] has shown that the soil fungus *Aspergillus terricola* contains enzymes acting upon many substances of biological origin. *Penicillium pinophilum* is one of the most active destroyers of cellulose yet isolated from soils, while *Aspergillus terricola* contains little if any cytase.

In general, the methods of culture and of preparing the enzyme powder used by Scales were applied also in our study of this *Penicillium*. The fungus mats on the surface of the nutrient solutions were prepared for the tests by the *Acetonedauer* treatment. A modified neutral Raulin's solution was used, ammonium sul-

[1] Scales, F. M., *J. Biol. Chem.*, 1914, xix, 459.

fate or peptone being employed as a source of nitrogen. **Pure cellulose** (acid-washed filter paper) was supplied as a source of carbon. The fungus often produced a dense mat on the surface of the liquid, the under side of this mat showing bright crimson spots. The cellulose cultures were incubated at about 28°C. for 2 to 3 weeks. During this time the cellulose had been changed so that it appeared soft and pulpy, the individual fibers being frayed out, separated, and transparent.

Some of the enzyme preparations made by the acetone treatment were from cultures containing purely inorganic nutrients, some from media containing peptone, while one was allowed to autolyze for 5 days with toluene as an antiseptic. Our preparations were gray or brown in color and the most active one contained 4.6 per cent of nitrogen. Definite amounts of the enzyme powders were weighed, mixed with distilled water, and allowed to stand for a time before being tested for their activity. Boiled enzyme solutions were used in every case as controls. The following enzymes were found to be especially active: sucrase, emulsin, and amidase; while diastase, catalase, and lipase were not as active. Tests for peptic, tryptic, ereptic enzymes, and also peroxidase, gave negative results. However, we should not conclude from this that proteolytic enzymes are absent in the growing fungus, because it is not likely that an active organism of this sort would be unable to attack protein. The activity of sucrase, diastase, lipase, and amidase was determined by quantitative methods upon various preparations of the enzyme powder. Space does not permit the inclusion of the results of our quantitative determinations in this study.

The most active preparation was made from the growth for 3 weeks on the inorganic nutrient solution containing ammonium sulfate and cellulose as the source of nitrogen and carbohydrate, respectively. Furthermore, this culture was just at the stage of active spore formation. In many cases the culture fluid from the older cultures showed a weak enzyme activity, indicating that some of the enzymes had diffused out of the cells. A study of the cytase of this organism was impossible at the time owing to the lack of methods for determining small quantities of cellulose with accuracy.

THE PROTEIN OF THE JACK BEAN CANAVALIA ENSIFORMIS.

By CARL O. JOHNS and D. BREESE JONES.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

The globulin was obtained by extracting with distilled water or dilute sodium chloride solutions and dialyzing the filtered solution in running water. The average figures for five different preparations are, carbon 52.36, hydrogen 6.90, nitrogen 16.29, sulfur 0.40, oxygen 24.05 per cent. When an extract is made as above and acidified with 0.2 per cent hydrochloric acid, the figures are the same except in the case of nitrogen which is nearly 1.0 per cent lower. When an extract made by means of distilled water is saturated with carbon dioxide the figures are the same as those obtained from dialysis. Further work is in progress.

THE DISTRIBUTION OF QUERCIMERITRIN IN THE COTTON PLANT (GOSSYPIUM HERBACEUM).

By ARNO VIEHOEVER, LEWIS H. CHERNOFF, and CARL O. JOHNS.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

Quercimeritrin has hitherto been isolated only from the petals of the cotton plant. The authors have found that it also occurs in the calyx and leaves of the cotton plant. The quercimeritrin was isolated by the method of Perkins. It was identified by its melting point and by the fact that it gave quercitin on hydrolysis.

CEDRIN, A GLUCOSIDE FROM THE SEEDS OF SIMABA CEDRON.

By ARNO VIEHOEVER, G. A. GEIGER and CARL O. JOHNS.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

Cedrin, the bitter principle of *Simaba Cedron*, has been used in medicine for some time, but seems to have received little attention from a chemical point of view. The authors obtained it by extracting the seeds of *Simaba Cedron* with 95 per cent alcohol. It crystallized in truncated rhombohedrons which melted with

decomposition at 265°C. Analysis gave carbon 61.74 per cent, hydrogen 6.46 per cent. It failed to give a test for nitrogen. The molecular weight in chloroform was 406. These figures agree well with $C_{21}H_{26}O_8$. On hydrolysis cedrin gave a sugar which has not been identified. Solubility determinations of cedrin were made.

A SAPONIN FROM YUCCA ANGUSTIFOLIA.[1]

By ARNO VIEHOEVER, LEWIS H. CHERNOFF, and CARL O. JOHNS.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

A saponin was isolated from the root stock of *Yucca angustifolia*. This gave a molecular weight in phenol of 773. It contained 52.97 per cent carbon, 6.77 per cent of hydrogen, and 40.26 per cent of oxygen. These figures agree closely with $C_{36}H_{56}O_{20}$, which is suggested as a provisional formula. On hydrolysis a sugar was obtained which gave the reactions characteristic of galactose.

A SAPONIN FROM YUCCA RADIOSA.[1]

BY CARL O. JOHNS, G. A. GEIGER, and ARNO VIEHOEVER.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

A saponin has been isolated from *Yucca radiosa* by extracting the ground stem with alcohol. In phenol it gave a molecular weight of 804. Analysis gave carbon 54.01 per cent, hydrogen 7.06 per cent, and oxygen 38.93 per cent. These figures agree with $C_{37}H_{58}O_{20}$, which is suggested as a provisional formula. On hydrolysis a sugar was obtained. Reactions indicate that this is glucose or mannose. Attempts will be made to crystallize this sugar.

INVESTIGATION OF THE KJELDAHL METHOD FOR DETERMINING NITROGEN.[1]

By I. K. PHELPS and H. W. DAUDT.

A study of various conditions of digestion of the Kjeldahl-Gunning-Arnold method was made on pyridine zinc chloride. It was found that mercuric oxide could not be replaced by various other catalysts and that the use of sodium sulfate in the place of potassium sulfate gave unreliable results. Experiments made with specially constructed lead condensers, using varying proportions of potassium sulfate and sulfuric acid showed complete decomposition of the pyridine when 0.3 gm. of pyridine zinc chloride was digested for 2½ hours in an open flask with a boiling mixture of 25 cc. of concentrated sulfuric acid, 10 gm. of potassium sulfate, and 0.7 gm. of mercuric oxide. The same proportions of reagents under the same conditions were found to give reliable results of the quantity of nitrogen present in these classes of cyclic compounds: pyrrole, pyrrolidine, pyridine, piperidine, quinoline, isoquinoline, purine, imidazole, quinoxaline, and quinazolone. Incomplete decomposition was found to result in the case of pyridine when the proportion of potassium sulfate was not sufficiently high.

THE NITROGEN DISTRIBUTION IN JACK BEANS, COW-PEAS, AND WHEAT.[2]

By J. F. BREWSTER.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

The nitrogen distribution in jack beans, cow-peas, and wheat were determined by the Van Slyke method.

A NEW SUGAR FROM THE AVOCADO (MANNOKETOHEPTOSE).[3]

By F. B. LA FORGE.

(From the Bureau of Chemistry, United States Department of Agriculture, Washington.)

The substance occurs free in the pulp of the ripe fruit, from which it was obtained by extraction with water. The extract was concentrated and freed from gum by precipitation with

alcohol. An heptatomic alcohol (perseit) crystallized from the concentrated alcoholic solution, leaving the sugar. From the syrup obtained by evaporation, the bromophenylhydrazone was prepared (m. p. 178°). The analysis of this as well as that of the osazone (m. p. 200°) shows the sugar to be a heptose. Cleavage of the bromophenylhydrazone with benzaldehyde yielded the free sugar which crystallized from dilute alcohol in six-sided prisms (m. p. 152°, $[\alpha]_D^{20} = + 29.5$). The sugar is non-fermentable and the fact that bromine is without oxidizing effect indicates that it is a ketose. The osazone appears to be identical with that of mannoheptose.

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[10] Presented from the Physiological Society in joint session of the Federation.

[11] Presented from the Pharmacological Society in joint session of the Federation.

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THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER
MEMORIAL FUND

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THE OCCURRENCE AND DETERMINATION OF CREATINE IN THE URINE.

By F. H. McCRUDDEN and C. S. SARGENT.

(*From the Laboratories of the Robert B. Brigham Hospital, Boston.*)

Some months ago we began to find creatine with surprising frequency in the urine of different patients, some of whom had shown no creatine on previous occasions. The fact that in all cases in which creatine was found the determinations were made by Folin's new method[1]—conversion of the creatine to creatinine by boiling with picric acid—whereas the earlier determinations had been made by the old method—in which the conversion is brought about by boiling with hydrochloric acid—made us suspect the method. Various control experiments were, therefore, made in an attempt to clear up the difficulty, the result of which is that we have been forced to conclude that the new method is not reliable; and that something other than creatine which gives the creatine reaction occurs in almost every human urine.

Experiments with Urine Alone.

1. A woman, slightly incapacitated with hypertrophic arthritis but otherwise well, was put on a diet free from meat, fish, soup, peas, beans, tea, and coffee, and containing chiefly eggs, milk, bread, rice, vegetables, and salads. The urine was found to contain 1.019 gm. creatinine in 24 hours.

3 cc. of urine, from a 24 hour amount of 3,000 cc., were treated with 20 cc. of a saturated solution of picric acid and 1.5 cc. of a 10 per cent sodium hydroxide solution and allowed to stand 10 minutes before being compared in a Duboscq colorimeter with a standard creatinine solution. The standard was 1 cc. of a 0.1 per cent solution of pure creatinine to which 20 cc. of a saturated solution of picric acid and 1.5 cc. of a 10 per cent solution of

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[1] Folin, O., *J. Biol. Chem.*, 1914, xvii, 469.

sodium hydroxide had been added; this was allowed to stand 10 minutes before being compared with the unknown. In these and the following determinations the standard was set at 20.0.

The readings of the unknown were as follows:

	1.	2.
	20.1	19.4
	19.3	19.2
	20.2	19.2
	20.4	19.8
	19.9	19.9
	18.6	19.5
Average,	19.75	19.5

These, when calculated for the total urine, give 1.012 and 1.026 gm. creatinine, an average of 1.019 gm.

2. The sum of the creatinine plus creatine in this urine was then determined by Folin's older method. It was found to be the same as the amount of creatinine alone, 1.020 gm. Creatine was, therefore, absent.

30 cc. of urine and 15 cc. of approximately N hydrochloric acid were heated on the water bath for 3 hours, cooled, and diluted in a volumetric flask to 50 cc. 5 cc. of this mixture—equivalent to 3 cc. of urine—were taken for the colorimetric determination; this was neutralized with sodium hydroxide; picric acid and alkali were added, and the colorimetric determination was carried out in the same way as before.

Duplicate determinations gave the following readings:

	1.	2.
	19.8	20.2
	19.0	19.6
	18.8	19.8
	19.8	19.5
	19.5	20.2
	19.3	19.4
Average,	19.4	19.8

These show respectively 1.030 and 1.010 gm. creatinine, an average of 1.020.

3. The sum of creatinine plus creatine was then determined by the new method. The amount found was 1.133 gm. According to this method the urine contains, therefore, 1.133 minus 1.019 or 0.114 gm. creatine.

To 3 cc. of urine were added 20 cc. of a saturated solution of picric acid and 130 cc. of water; the solution was boiled gently for 1 hour, after which

it was rapidly boiled down to 20 cc., then cooled, and 1.5 cc. of a 10 per cent sodium hydroxide solution were added to it. After 10 minutes the color was compared with that of the standard.

The readings were:

1.	2.
17.6	17.4
18.2	17.5
18.0	17.5
17.8	17.6
17.8	18.3
17.6	17.0
Average, 17.8	17.55

These give 1.126 and 1.140 gm., respectively, of creatinine plus creatine (average 1.133 gm.).

4. A determination of the creatine by the new method was then made on the specimen which had been boiled with hydrochloric acid for determination of creatine by the old method (see 2); this determination gives, therefore, the results found by superimposing the new method on the old method. The amount found was 1.158 gm. or 1.158 minus 1.019, equal to 0.139 gm. of creatine, practically the same as found by the new method alone.

5 cc. of the mixture used in 2 were boiled for 1 hour with 20 cc. of a saturated solution of picric acid and 130 cc. of water, then evaporated quickly to 20 cc. and cooled; 1.5 cc. of a 10 per cent sodium hydroxide solution were added and the color obtained was compared with that of the standard.

Duplicate determinations were made and the average of six readings was taken in each case as before.[2] The average readings were 17.05 and 17.55, respectively. This gives 1.173 and 1.143 gm. creatinine plus creatine, an average of 1.158.

These figures are but examples of those obtained in numerous analyses, the limits of error of which cannot be more than 0.02 to 0.03 gm. creatinine. This seems to indicate that the results obtained by the new method are not due simply to a more complete conversion of creatine into creatinine by the new method, but to the presence in the urine of some compound other than creatine which gives a color reaction on boiling with picric acid, but not in the cold; and which does not change to creatinine on boiling with hydrochloric acid.

[2] In these and all the following experiments at least six readings were taken in every case, just as in 1, 2, and 3.

Experiments with Pure Solutions.

On showing our results to Dr. Folin he very generously offered us, for the purpose of testing the matter further, the specially purified creatine and creatinine prepared in his own laboratory; and in the following experiments these were used.

Analyses 5, 6, and 7 were carried out to determine whether pure creatine reacts differently by the two methods, the old and the new; and whether creatinine itself gives higher results on boiling with picric acid than it does in the cold.

5. Two 1 cc. specimens of a 0.1 per cent solution of pure creatinine were boiled for 1 hour with picric acid as in the new method. Comparison with the standard showed an average of 1.029 mg. creatinine,[3] an amount which is within the limits of error.

6. To this same 0.1 per cent solution of creatinine enough pure creatine was added to make the solution contain 0.015 per cent creatine (equivalent to 0.013 per cent creatinine). Analyses of the solution by the old method showed 1.157 mg. creatinine plus creatine, or 1.157 minus 1.029, equal to 0.128 mg. creatine in 1 cc. (instead of the 0.130 mg. present).

7. Experiment 6 was repeated using the new method instead of the old method for converting creatine into creatinine. The result was the same, 0.128 mg. creatine.

The results show that creatinine itself does not give higher results on boiling with picric acid than it does in the cold; and that creatine is converted quantitatively into creatinine on boiling with hydrochloric acid. The inference drawn from the earlier experiments, namely, that the deepening of the color on boiling urine with picric acid and then adding sodium hydroxide, is due to something other than creatine, is confirmed by these experiments.

Experiments with Pure Solutions plus Urine.

In order to see if conditions in the urine alter the behavior of creatine, experiments similar to the preceding three were repeated on urine to which pure creatine had been added. For purposes of description we will refer to real creatine here as *a*-creatine and

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[3] In these and the succeeding experiments duplicate determinations were always made; the figures given show the average result.

to the supposed new substance or substances responsible for the reaction on boiling with picric acid as x-creatine; we do this merely for convenience and do not thereby mean to imply that x-creatine has any chemical relationship to a-creatine or even that it is a single substance. The urine used in these experiments was the same as that used in Experiments 1 to 4; it contained 1.019 mg. creatinine, no a-creatine, and 0.138 mg. x-creatine (calculated as creatinine) in 3 cc.

8. 0.150 mg. creatine was added to 3 cc. of the urine and the creatinine plus creatine determined by the old method. The amount found was 1.163 mg. This gives 1.163 minus 1.019, equal to 0.144 mg. creatine.

9. In the same solution of creatine in urine used in the preceding experiment, creatinine plus creatine was determined by the new method. The amount found was 1.282 mg. Subtracting from this the creatinine plus x-creatine present, as found in Experiment 3, we get 0.124 mg. of a-creatine (calculated as creatinine) which is practically the amount added (0.130 mg.).

The results show that the conditions occurring in the urine do not affect the behavior of creatine in such a way as to account for the results.

Experiments with Other Reducing Substances.

The possibility that the results might be due to the presence of other reducing substances led us to try the effect of small amounts of glucose and uric acid on the reaction.[4]

The urine used in these experiments was free from any reducing substances detectable by the ordinary methods; but since glucose does give a color reaction on boiling with alkaline picrate we tried the effect of such quantities of glucose and uric acid on the reaction as might occur in normal urine.

A 300 cc. specimen of urine was divided into three portions, a, b, c, of exactly 100 cc. each. To a nothing was added; to b 30 mg. of pure glucose; to c 30 mg. pure uric acid. Creatinine, a-creatine, and x-creatine were then determined on all three specimens.

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[4] The statements of Folin (*J. Biol. Chem.*, 1914, xvii, 473) and S R. Benedict (*J. Biol. Chem.*, 1914, xviii, 191) regarding the effect of sugar on the reaction are somewhat conflicting.

The amount of creatinine found, calculated in mg. per 100 cc., was as follows:

	a	b	c
Creatinine	2.29	2.28	2.28
Creatinine plus creatine	2.39	2.41	2.35
(boiling with hydrochloric acid)			
Creatinine plus creatine	2.58	2.55	2.59
(boiling with picric acid)			

This urine—not the same as that used in the previous experiments—contains 0.10 mg. a-creatine and 0.20 mg. x-creatine. The results are not affected in the slightest degree by the glucose and uric acid added.

<div align="center">DISCUSSION.</div>

The figures show conclusively that there is something in the urine other than creatine which, on boiling with picric acid, gives a color reaction similar to the creatinine reaction. The results cannot be accounted for by assuming that the creatinine itself gives a deeper color on boiling with picric acid than it does in the cold (Experiment 5); they are not due simply to a more complete change of the creatine into creatinine on boiling with picric acid than on boiling with hydrochloric acid (Experiments 6 and 7); they are not due to a partial destruction of the creatine on boiling with hydrochloric acid (Experiment 4).

Since the new method shows creatine in amounts of 0.1 to 0.2 gm. per day in almost every urine, whereas the old method shows no creatine in normal urine, but does show it in the urine in cases of various muscular dystrophies, starvation, diabetes, and other conditions where we might expect it to be found, we can say that in the present state of our knowledge of creatinine and creatine metabolism, the results obtained by the old hydrochloric acid method are probably more significant than those obtained by the new picric acid method.

The amount of x-creatine found is variable; we have not observed that it shows any relationship to the patient's weight or to the diet. The following table shows the amounts of creatinine, a-creatine, and x-creatine in gm. per day found in two nearly normal patients. The table gives fair examples of the amounts of x-creatine ordinarily found; it is occasionally absent from the urine.

Day.	Woman.			Man.		
	Creatinine.	a-Creatine.	x-Creatine.	Creatinine.	a-Creatine.	x-Creatine.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.651	0	0.130	1.924	0	0.161
2	0.680	0	0.107	1.929	0	0.402
3	0.653	0	0.141	1.975	0	0.190
4	0.655	0	0.073	1.989	0	0.102
5	0.650	0	0.173	1.970	0	0.209
6	0.633	0	0.167	1.964	0	0.154

The following table shows the figures obtained in a case of progressive muscular dystrophy where real creatine was present as well.

Day.	Creatinine.	a-Creatine.	x-Creatine.
	gm.	*gm.*	*gm.*
1	1.483	0.283·	0.012
2	1.503	0.316	0.091
3	1.481	0.599	0.072
4	1.493	0.624	0.007
5	1.486	0.578	0.060
6	1.484	0.409	0.110
7	1.471	0.448	0.068

These results were obtained several months ago. We had then hoped to find time to isolate the substance or substances which we have called x-creatine. The necessity of postponing further work on the subject for some time to come makes it seem advisable to publish our findings in order to call to the attention of other workers the inaccurate conclusions which might be drawn on finding so called creatine in the urine by this method.

SUMMARY.

Human urine contains a substance or substances other than creatine which can give a color reaction similar to that of creatinine on boiling with picric acid, and which, therefore, may appear in the results as creatine.

A CRITIQUE OF CERTAIN DATA ON THE CONTENT OF CHOLESTEROL AND FATTY SUBSTANCES IN THE BLOOD, TOGETHER WITH A MODIFICATION OF THE COLORIMETRIC METHOD FOR ESTIMATING CHOLESTEROL.

By FRANK A. CSONKA.

(*From the Laboratory of Dr. J. P. McKelvy, Pittsburgh.*)

Since the report by Faust and Tallquist[1] of their studies on the hemolytic action of the unsaturated fatty acids and their soaps, attention has been drawn to the possibility of these substances exercising an important rôle in the grave anemias.

King[2] and Medak,[3] in collaboration with Eppinger, have recently published analyses of the blood lipoids in different diseases and found in the severe anemias an exceptionally high iodine number, which they considered to be characteristic, and which suggested that the hemolysis was due to the unsaturated fatty acids. But their method and calculations are such as to make all their results questionable. I have found the analytical method followed by King in Medak's publication to be, in detail, as follows:

Es wurden 100 ccm durch Venae punctio entnommenen Blutes defibriniert und mit 1000 ccm 96%igem Alkohol versetzt. Auf diese Weise wurden die Eiweisskörper quantitativ gefällt. Ein Teil der Blutfette ging hierbei in den Alkohol über. Der gesamte Niederschlag wurde vom Alkohol abfiltriert und getrocknet, das Filtrat eingedampft. Der auf dem Filter verliebene, getrocknete Niederschlag und der Abdampfrückstand wurden im Soxhletapparate mit Petroläther (Kahlbaum) durch 72 Stunden extrahiert. Der Petroläther wurde abgedampft, der auf Gewichtskonstanz gebrachte Rückstand ergab die Menge des in 100 ccm Blut enthaltenen Gesamtfettes. Der Rückstand wurde nun mit heissem 96%igen Alkohol

[1] Faust, E. S., and Tallquist, T. W., *Arch. exp. Path. u. Pharm.*, 1907, vii, 367.

[2] King, J. H., *Arch. Int. Med.*, 1914, xiv, 145.

[3] Medak, E., *Biochem. Z.*, 1914, lix, 419.

extrahiert, der Extrakt auf ungefähr 50 ccm eingeengt und mit heisser
Digitoninlösung (Digitonini cryst.—Merck—1.0: Alkohol 96% 100.0) das
Cholesterin ausgefällt, der Niederschlag auf einen vorher gewogenen, mit
Asbestwolle beschickten Goochtiegel gebracht, getrocknet und gewogen.
Die erhaltene Zahl, durch 4 dividiert, ergab die Menge freien Cholesterins.
Nun wurde das beim Abfiltrieren des Digitonincholesterids erhaltene alko-
holische Filtrat mit dem auf dem Goochtiegel verbliebenen Niederschlag
vereinigt und mit Petroläther extrahiert . . . . ''

Why extract with petroleum ether, after weighing? Or, if
the petroleum ether extraction is necessary, and I believe it is,
to get the digitonin cholesterids free from lipoid substances, why
not weigh after extraction? The first value is certainly incorrect.

''   .   .   .   .   der Extrakt eingedampft, der Rückstand in 96%igem Alko-
hol gelöst und mit Natriumäthylat verseift. Auf diese Weise wurde das an
Ester gebundene Cholesterin frei. Nach der Verseifung wurde filtriert
[?], das Filtrat [A] abermals mit Petroläther mehrmals extrahiert, der
Extrakt eingedampft, in heissem 96%igen Alkohol gelöst und mit heisser
1 %iger alkoholischer Digitoninlösung gefällt, der Niederschlag auf einen
vorher gewogenen Goochtiegel gebracht [B], getrocknet und gewogen. Die
erhaltene Zahl, durch 4 dividiert, ergab die Menge an Ester gebundenen
Cholesterins. Das Filtrat wurde zur Bestimmung der Hüblschen Jodzahl
verwendet.''

It is not made clear, but it is likely, that the filtrate designated
A and not B was used for the iodine number determination. It is
wrong to disregard B entirely because a small amount from the
soap of the unsaturated fatty acids goes into the petroleum ether
as an impurity. The bulk of the fatty acids existing as soap is
in filtrate A. Before extraction, it has to be liberated by acidifi-
cation, as was done by Medak, but King extracted these soaps
after evaporation to dryness, by chloroform.

It is, of course, possible that the hemolysis in anemia is due to
unsaturated fatty acids, but as the residue, in which King deter-
mined the iodine number, contained fatty acids only as impurities,
there seems little basis for the hypothesis that the high iodine
numbers were due to unsaturated fatty acids. But one may say
that the unknown unsaponified material, which was dissolved
by chloroform extraction, contains the substance, which absorbed
the iodine and gave the high iodine number. This would be true
if the calculation had been correct. To demonstrate this error,
I have to again cite from the original article:

"King bestimmte die Jodzahl in folgender Weise: Das Filtrat, das beim
Abfiltrieren von dem an Ester gebundenen Cholesterin erhalten wurde,
wurde eingedampft, in heissem Chloroform gelöst und auf 100 ccm auf-
gefüllt. 10 ccm davon wurden mit 25 ccm der Hüblschen Jodlösung versetzt
. . . . Die Jodzahl erhielt King aus einem Bruche, in dessen Zähler
die Zahl der verbrauchten Kubikzentimeter Natriumthiosulfatlösung,
multipliziert mit dem Titer der letzteren, in dessen Nenner eine Zahl
stand, die sich aus der Differenz zwischen Gesamtfett und freiem Choles-
terin + an Ester Gebundenem Cholesterin ergab. Stets wurden Choles-
terin, Cholesterinester und Jodzahl auf 1000 ccm Blut berechnet."

When we consider that the iodine number represents the amount
of iodine in gm. absorbed by 100 gm. of fatty material, it is appar-
ent that the calculations of King and Medak are incorrect. King
cites in his paper the iodine value of oleic acid as 90, the true
iodine number of oleic acid by the usual method of calculation.
Yet we find in Medak's quotation[3] of King's work, the iodine
number calculated from the number of cc. of 0.1 N $Na_2S_2O_3$ used,
divided by the amount of fatty substance in gm., on the basis of
1,000 cc. of blood. King does not mention this calculation in
his paper, but only gives the result, leaving the nature of the
unsaturated material in doubt.

It is not possible to compare results obtained by two so widely
different methods of calculation, so that their high iodine numbers
(King 326 and Medak 629) are meaningless, in terms of the
usual method of calculation.

Further, I could not find any explanation why King and Medak
took only 10 cc. of the chloroform solution, when the reference
King mentions[4] advises taking 0.15 to 0.18 gm. of the substance
containing unsaturated fatty materials for the iodine number
determination. The entire 100 cc. of chloroform solution could
not contain more substance than the required quantity, and tak-
ing as little as in 10 cc. increases the possibility of error.

To avoid criticism of my results by the use of a different method
it was my intention to follow the procedure of King and Medak
as nearly as possible in obtaining the extract of the blood fat.
Regardless of how the blood is prepared for extraction, or what
solvents are used, the extraction is always incomplete by the
present methods. Even with the most effective. solvent ethyl
alcohol, followed by ether or petroleum ether, a small amount is

[4] "Abderhalden's Handb. biochem. Arbeitsmethoden, II."

always found in the residue from the fat and cholesterol. As the
isolation of the fat and cholesterol by.the saponification method
is quantitatively correct, it would eliminate the small error and,
if enough material is available, would seem preferable.

Methods.

The blood was taken from the vein 2 hours after a breakfast
of tea and toast (without milk or butter), except in Case 2, which
was taken 4 hours after a full meal. A few crystals of potassium
oxalate were added to prevent clotting. 100 cc. of blood were
diluted to 1,000 cc. with 95 per cent alcohol and, after standing
24 hours, were filtered directly into a large extraction shell and
extracted by absolute alcohol for 24 hours and then by petroleum
ether for 48 hours in a Soxhlet apparatus. The filtrate from the
blood protein precipitate, as well as the absolute alcohol and
petroleum ether extracts, was evaporated on the water bath to
dryness, dissolved again with petroleum ether, and filtered. In
the three anemia cases, this petroleum ether extract was evap-
orated and dried *in vacuo* over sulfuric acid to constant weight
(in Table I, fat + lipoids). In the hemolytic jaundice, the petro-
leum ether extract was diluted to 100 cc. and only 50 cc. were
used for determining the fat + lipoids. Windaus'[5] digitonin
method, as modified by Fraser and Gardner,[6] was used for the
cholesterol determination.

For the saponification of the esters of cholesterol, the method
as described by Gephart and Csonka[7] for fatty acids was used,
naturally without acidifying before extraction by ether (A). The
ether extract was washed with distilled water until neutral (B),
the ether extraction residue dissolved in alcohol, and the choles-
terol (combined) determined as before. The alkaline solution A,
containing the chief bulk of fatty acids, was acidified and extracted
with ether (first with the ether used for washing the cholesterol
digitonin—as combined—precipitate) and then combined with
the ether extract of the wash water B and the ether extract of the

[5] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.
[6] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1909-10,
lxxxii, 560.
[7] Gephart, F. C., and Csonka, F. A., *J. Biol. Chem.*, 1914, xix, 521.

wash water from the digitonin cholesterids (as combined); the two latter were also first acidified to liberate the fatty acids. The ether extract, after washing free from hydrochloric acid, was evaporated and dried *in vacuo* over concentrated sulfuric acid to constant weight (Table I, fatty acids). This dissolved in 10.0 cc. chloroform served for the iodine number determination according to Hübl.

In Case 4, as mentioned before, 50 cc. of the petroleum ether extract were used for the direct determination of the iodine number, as it was thought that the highly unsaturated fatty acids might have been destroyed by the saponification. It was found

TABLE I.

In 1,000 Cc. of Blood.

Case.	Fat + lipoids.	Cholesterol.			Fatty acids.	Iodine number on 100 gm. fatty acids.	Remarks.
		Free.	Combined.	Total.			
	gm.	gm.	gm.	gm.	gm.		
1	4.7860	0.2110	0.5820	0.7930	2.284	53.36	Pernicious anemia.
2	6.9730	0.6448	0.8144	1.4592	4.934	56.43	" "
3	5.6575	0.5759	0.7129	1.2888	2.937	70.43	" "
4	6.9100	0.7196	0.5144	1.2340	2.500	103.30	Congenital hemolytic jaundice.

that 0.3455 gm. extract absorbed 0.2618 gm. iodine, giving an iodine number of 75.76; but 0.0405 gm. iodine was used by 0.0617 gm. cholesterol present. Therefore, the iodine number of

the extract minus the cholesterol $\left(\dfrac{0.2213 \times 100}{0.2838} \right)$ is 98.17, corre-

sponding to the iodine number of the fatty acids of 103.3 (see Table I) obtained after saponification. Thus, either by the direct determination or after saponification we get in congenital hemolytic jaundice an iodine number only a little higher than that of oleic acid. To determine the nature of the substance which is responsible for the absorption of iodine a further isolation is necessary. This project will be reported later.

As pointed out in the introduction, the extraction of the blood by any combination of the best solvents is never complete. To demonstrate this, 10 cc. of the whole blood were saponified, as described by Gephart and Csonka,[7] for the fatty acids, and

another 10 cc. were used for the total cholesterol determination, by extraction from the alkaline solution. On the other hand, two samples were taken of the dried blood protein residue after extraction (obtained from 100 cc. blood) and saponified, one for cholesterol and the other for the fatty acids determination. The results are given in Table II.

The form in which the cholesterol was present, free or combined, could not be determined, but it represents, in the extraction method of cholesterol determination as in that of the fatty acids, an error of over 10 per cent.

Though the results will be published later when more cases of other pathological conditions are collected, I wish to describe a modification of the Grigaut[8] and Autenrieth-Funk[9] colorimetric

TABLE II.

In 1,000 Cc. of Blood (Case 4).

	Extract.	Residue.	Total.	Saponifi-cation.	Colorime-tric.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Fatty acids.............	2.500	0.3276	2.8276	2.7646*	
Cholesterol (total)......	1.2340	0.1432	1.3772	1.4330	1.415

*0.1 N cc. × 0.0274.

methods of estimation of blood cholesterol, a method used success- fully in this laboratory during the past year.

2 cc. of blood measured by an Ostwald pipette are placed in a flask (Fig. 1). 20 cc. of alcohol (95 per cent), 4 gm. of potassium hydroxide, and 2 cc. of a 10 per cent solution of barium chloride are added. The contents are boiled under a reflux condenser for 1 hour, cooled, distilled water is added to the constriction (about 50 cc.), and 50 cc. of ether are added. The flask is closed with the stopper and shaken in a rotary manner for a few minutes. After the ether layer has separated it is blown by pressure into a separating funnel (250 cc.). The ether extraction is repeated twice with 50 cc. portions, collecting the portions in the sepa- rating funnel. After each extraction the ether layer is brought to the constriction by adding a little distilled water. The com-

ʼ Grigaut, A., *Compt. rend. Soc. biol.*, 1911, lxxi, 513.
ʼ Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

bined ether extract in the separating funnel is washed with
several portions of water until the wash water is neutral. The
turbid ether extract after being filtered into an Erlenmeyer flask
is perfectly clear and colorless. After the ether is distilled off
the flask is placed in a water bath for half an hour. When the
flask is cooled off, the residue is dissolved in 3 cc. portions of
chloroform (at room temperature), each portion is filtered through
a 5.5 cm. diameter filter paper, and collected in a 10 cc. normal
flask. The flask is filled up to the mark with chloroform and 5
cc. are placed in another 10 cc. normal flask to serve as a control.
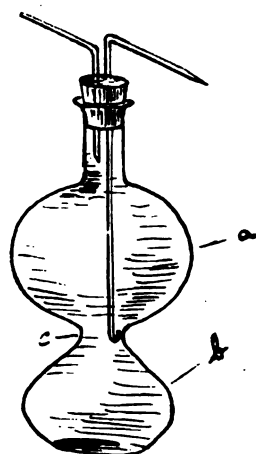
Fig. 1. a. Upper bulb, about 130 cc.
b. Lower bulb, 70 cc.
c. Constriction, 25 mm. diameter.

A cholesterol solution containing 1 mg. of pure cholesterol in 5 cc.
of chloroform is used as a standard. 2 cc. of acetic anhydride
and 0.1 cc. concentrated H_2SO_4 are added to each of the flasks,
which are placed in a dish of water (30°C.), and let stand in the
dark for 15 minutes. After this the flasks are cooled rapidly,
filled with chloroform to the 10 cc. mark, and the color is
compared immediately by the Duboscq colorimeter, setting the
standard at 15 mm. It is necessary to use tubes with screw
attachment.

The above described procedure of adding $BaCl_2$ to the saponi-

fication gives a very satisfactory reading as it eliminates almost entirely the disturbing yellowish color.

SUMMARY.

In three cases of pernicious anemia the fatty acids of blood gave an average iodine number of 60. In one case of congenital hemolytic jaundice the iodine number 103.3 was found.

The high iodine number in pernicious anemia and congenital hemolytic jaundice reported by King and Medak is based on incorrect calculation.

The blood cholesterol in all four cases is considerably lower than that of a normal individual, which agrees with the findings of previous authors.

A modification of the colorimetric determination is presented.

DETERMINATION OF CREATINE IN MUSCLE. II.

By LOUIS BAUMANN and HARRY M. HINES.

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From the results of their experimental work, Janney and Blatherwick[1] conclude that "the principle of hydrolysis with acids is to be avoided in the estimation of creatine in complex tissues." They recommend an extraction method which they claim to be accurate and simple. A comparison of the figures obtained by this method with those obtained by an acid hydrolysis method, reported by one of us two years ago[2] fails to show any marked difference between the two. In the case of dog muscle, which was chiefly used by Janney and Blatherwick for their determinations, the agreement is striking. The average for dog muscle (sixteen different animals) corresponds with that reported by Myers and Fine.[3]

The older method has now been simplified so that a complete determination may be carried out in less than $\frac{1}{2}$ hour after the extract is obtained. This method may be used with advantage for the estimation of the sum of creatine and creatinine in organs. The results when expressed in terms of 100 gm. of moist organ are a few mg. higher than those obtained by the Janney and Blatherwick method.

The apparent objections to the Janney and Blatherwick method are that it is occasionally difficult to obtain a representative 5 gm. sample and also that filtration of certain organ extracts according to this method is extremely slow.

It is a matter of considerable importance to know if the Jaffé reaction as it is obtained with the hydrolyzed muscle extract is

[1] Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.

[2] Baumann, L., *J. Biol. Chem.*, 1914, xvii, 15.

[3] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xiv, 9.

to be attributed to creatinine alone. We attempted to precipitate this substance as the potassium picrate salt. We find that all the substance in the hydrolyzed muscle extract which gives thé Jaffé reaction may be precipitated in this way.[4] Janney and Blatherwick suggest that acetyl-propionic acid which also gives the Jaffé reaction, may be formed when muscle tissue is heated with solutions of mineral acids. When 50 gm. of muscle together with 0.5 gm. of glucose are heated for 3 hours with 5 N sulfuric acid according to Baumann, no increase in the colorimetric reading is observed.[5]

EXPERIMENTAL.

Comparison of the Two Methods.

The creatine content is expressed in mg. per 100 gm. of moist muscle or organ.

Dog muscle.		Beef muscle.	
Janney and Blatherwick.	Baumann.	Janney and Blatherwick.	Baumann.
mg.	*mg.*	*mg.*	*mg.*
376	381	391	412
362	379	462	451
386	381	490	488
380	383	415	421
376	378	435	425
366	365	427	433
386	384	455	450
389	390	373	369 (much connective tissue.)
376	370	407	411
365	362	439	450
348	353	Average 429.4	431.0
353	353		
316	328		
379	371		
381	394		
367	365		
Average 369.1	371.0		

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[4] This work was carried out by Mr. Ingvaldsen. The quantitative data will appear in a subsequent article.

[5] 0.5 gm. of glucose is more than the sum of glucose plus glycogen in 50 gm. of muscle.

Cat muscle.		Rabbit muscle.	
Janney and Blatherwick.	Baumann.	Janney and Blatherwick.	Baumann.
mg.	mg.	mg.	mg.
472	477	500	485
492	497	537	543
497	504	547	548
Average 487	492.7	531	534
		Average 528.7	527.5

	Janney and Blatherwick.	Baumann.
	mg.	mg.
Dog brain....	108	120
Beef "	103	113
" testicles.	175	195
Sheep " .	203	215
" " .	216	209
Dog liver.....		28.2
" "		24.3
" "		23.6
" "		21.0

The Effect of Added Glucose to Muscle before Hydrolysis.

50 gm. of hashed beef muscle to which 0.5 gm. of glucose had been added were boiled for 3 hours under a reflux with 125 cc. of 5 N sulfuric acid and the creatinine was determined as described below. The control contained 433 mg. of creatine per 100 gm. of mu-cle, whereas the muscle plus sugar contained 431 mg.

The Simplified Method for the Determination of the Sum of Creatine and Creatinine in Muscle.

The technique is similar to that already described,[2] except that 10 cc. of the extract are used instead of 20 cc. This makes evaporation unnecessary. The procedure is briefly as follows: 10 cc. of the hydrolyzed and filtered extract are pipetted into a 50 cc. volumetric flask, 9 cc. of 10 per cent sodium hydroxide solution added, the solution is cooled, and then diluted to the mark with saturated picric acid solution. The contents of the flask are well mixed, then filtered. The creatinine is determined according to Folin. 25 cc. of the clear filtrate and the requisite amount of standard solution are pipetted respectively into 250 cc. volumetric

flasks.   15 cc. of saturated picric acid solution and sufficient dis-
tilled water to equalize the volumes in both flasks are added to
the standard.   The color is developed by adding 2.5 cc. of 10 per
cent sodium hydroxide solution to the standard and 3 cc. to the
unknown.   After 10 minutes the solutions are diluted to the mark
and the color intensities compared.   The standard is set at 10
mm.   The standard consists of a 0.1 N hydrochloric acid solu-
tion containing the equivalent of 1 mg. of creatinine per 1 cc. of
solution.   We have employed the creatinine zinc chloride salt
for this purpose.   For the determination of dog muscle 3 cc.,
for beef 3.5 cc., and for rabbit muscle 4.5 cc. of this solution are
required.

The results obtained by this procedure are practically identical
with those obtained by the older method.[2]

	Older method.	Newer method.
	mg.	*mg.*
	385	382
	388	383
	457	467
	384	389
	341	343
	—	—
Average	391	393

SOME INDICATORS FROM ANIMAL TISSUES.

By W. J. CROZIER.

Contributions from the Bermuda Biological Station for Research, No. 44.

Indicators occurring in nature have for the most part been found in plants; in fact such materials have seldom been recorded from animal sources. An examination of a number of pigmented animals, however, shows that this rarity is by no means so great as has been supposed. The following notes refer to prep-

TABLE I.

Color Changes of Some Indicators from Animal Tissues.

Source.	Acidity.[*]								
	p_H=5.0	5.3	5.6	5.9	7.4	7.6	7.65	8.0	10.0
Ascidia atra	Red.	(Increasingly red.) ←			Red.	Green (ppt.).			
Ptychodera sp							Yellow.	Brown.	
Chromodoris zebra			Pink.	Blue.	Blue.	Blue.	Blue (green ppt.).		
(?) *Eupolymnia aurantiaca*								Red.	Green.

[*] Observations were made at a number of intermediate points.

arations obtained from some marine animals, which give clear-cut[1] reversible color changes as summarized in Table I. The hydrogen-ion concentrations observed were obtained by acetate, borate, and phosphate mixtures, at 20–25°, with the usual indicators for comparison (Michaelis, 1914). The tests were made by

[1] A number of other soluble pigments have been found in which an *acid*-alkali color change is undoubtedly present, but is not sufficiently *clear*-cut to be of use. For example, a sponge, *Tedania ignis*, gives brown *extracts*, which are made distinctly red by acids.

adding two drops of a concentrated solution of pigment to 10 cc. of the different mixtures.[2]

1. Ascidia atra.—This common black tunicate of Bermuda and the West Indies contains in its test a substance readily extracted by acetone, less easily by alcohol or glycerol, but not dissolved by ether or chloroform. This pigment is for the most part closely packed in the outer tenth of the thickness of the test, and occurs in the form of rather large granules carried by cells in the test canals. Its color is a deep purple, as may be seen when prepared sections of the test are studied microscopically. The pigment is insoluble in sea water, but observations indicate that it is excreted in granular form from the surface of the animal. According to Dahlgrün (1901), in many tunicates an excretory function is maintained by wandering mesenchymal cells the protoplasts of which bear pigment granules.

Solutions of the pigment are dark red, this color being intensified by acids and turned green by alkalies, which also precipitate the substance in greenish flocculent masses.

There is probably some relation between this dark purplish red substance and the pigment, described by Herdman (1913) and Holt (1914), which occurs in the test of the compound ascidian *Diazona violacea*,[3] although the *Ascidia* pigment does not appear to show any absorption bands.

2. Ptychodera sp.—This yellowish balanoglossid when strongly stimulated secretes a yellowish material which turns reddish brown at about $p_H = 8.0$. Extracts of the animal, containing this pigment, can be made with alcohol, distilled water, or aqueous solvents, but they are not useful as indicators because the brown color is permanently assumed on standing for some time. But

[2] Useful indicators have also been prepared from several Bermuda plants, as the berries of the sage bush (*Lantana involucrata*), which yield on extraction with alcohol a bright red solution, changing to yellow (alkaline) at $p_H = 6.6$; the fruit of the cactus (*Opuntia*) also contains a red material changing to lemon yellow (alkaline) at $p_H = 9.0$; the juice from the ink berry of the sand beaches (*Randia aculeata*) is a splendid indicator, being dark red with acids and changing to a brilliant green (alkaline) at $p_H = 8.5$.

[3] Coloring matter precisely like that of *Diazona violacea* occurs in Bermuda ascidians which are closely related to this species.

within the tissues of the living animal the change resulting from the penetration of alkalies is well shown.

3. Chromodoris zebra.—The indicator contained in this nudibranch has been described elsewhere (Crozier, 1914, 1915, 1916), but for comparison its color changes have been included in Table I.

4. Eupolymnia aurantiaca (?).—An orange red annelid, probably *Eupolymnia aurantiaca*, which is occasionally found among blocks of coral rock in which it burrows, gives with aqueous alcohol a bright red extract of the integument, which changes to orange on standing for some days. It shows a sharp transition from red to bright green at about $p_H = 10.0$, though even at higher acidities a precipitate (which consists of the pigment substance) appears slowly; but the color change in the solution appears only above $p_H = 10.0$. The red solutions transmit only a narrow portion of the red and of the yellow, while the green acid solutions transmit only the green-blue.

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FAT ASSIMILATION.

By W. R. BLOOR.

(*From the Laboratories of Biological Chemistry of the Harvard Medical School, Boston.*)

Our conception of the changes which ingested fat undergoes in the animal body is fairly clear as regards the beginning and the end but very vague as to the intermediate stages. The way in which the food fat (or at least the greater part of it) gets from the intestine into the blood has been quite satisfactorily determined. It is saponified in the intestine, absorbed in water-soluble form as soaps and glycerol, resynthesized by the intestinal cells, and passed into the chyle and thence to the blood as neutral fat (glycerides) suspended in the plasma in a very finely divided condition. About 60 per cent of the food fat has been actually accounted for in the chyle in this way and this figure is probably low. The remaining smaller quantity is generally assumed to be absorbed directly into the blood stream by way of the intestinal capillaries. Support is given to this assumption by the work of Munk and Friedenthal[1] who found that the blood fat increased after the thoracic duct had been ligated, also by the finding of d'Errico[2] that the fat content of the portal vein is always higher than that of the jugular during fat absorption, and by the work of Hamburger[3] who found that the absorption of soaps from an isolated loop of intestine took place after all visible lymph vessels had been tied off.

There is also a quite satisfactory hypothesis regarding the later stages of fat metabolism based on the work of Knoop[4] and of

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[1] Munk, I., and Friedenthal. H., *Zentr. Physiol.*, 1901, xv, 297.

[2] d'Errico, G., *Arch. fisiol.*, 1907, iv, 513.

[3] Hamburger, H. J., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1900, 554.

[4] Knoop, F., *Beitr. chem. Phys. u. Path.*, 1905, vi, 150.

Leathes and his coworkers.[5] The long chain fatty acids are desaturated in the liver and are then broken down to carbon dioxide and water by successive oxidations. Whether the long chains are broken into shorter ones at the points of desaturation previous to oxidation is a disputed point. At some stage in the oxidation carbohydrates appear necessary for complete combustion.

But as to what happens to the fats from the time they enter the blood stream until they reach the desaturation stage we have very little information of any kind. The suspended fat persists in the blood for some hours and then disappears. Some part of it goes to the liver, whose fat content increases during fat absorption. The blood serum 10 to 12 hours after a meal is clear and free of suspended particles, and remains so during fasting, although considerable quantities of fat must be passing from the fat stores into the blood for the use of the organism, especially after the 2nd or 3rd day of the fast, when the energy requirement is supplied mainly by the stored fat. Because of the fact that the serum remains clear during the transference it is probable that the fat is not being carried as such but as some derivative more readily miscible with water, possibly lecithin.

Ever since the chemical similarity between lecithin and the fats was discovered, attempts have been made to connect the two in metabolism; but although there has been much speculation no definite evidence has been offered. In fact lecithin has been generally classed as a cyto-lipoid, having to do more with the structure of the cell than with its processes. However, the idea that there is a dynamic relation between lecithin and fat in normal metabolism has been a persistent one and has continued to stimulate investigation so that recently evidence has been brought forward[6] definitely connecting the two. The evidence consisted of fat absorption experiments in which it was found that lecithin increased in the blood during the absorption of the fat. It seemed probable at the time, in view of the work of Leathes and others, that the seat of lecithin formation was the liver, in which case the

[5] Leathes, J. B., and Meyer-Wedell, L., *J. Physiol.*, 1909, xxxviii, p. xxxviii. Hartley, P., *J. Physiol.*, 1909, xxxviii, 353.

[6] Bloor, W. R., *J. Biol. Chem.*, 1915, xxiii, 317.

observed increases in lecithin would probably be more marked in the plasma than in the whole blood, since the plasma is ordinarily regarded as the carrier.

The present work is a continuation of the above, carried out in order to get further information regarding the process and to locate if possible the seat of formation of the lecithin.

Dogs were given a feeding of fat only, with the exception of Experiment 4 where water was given. Blood samples (10 cc.) were taken at once and at 2 hour intervals for 8 hours. Comparative analyses of whole blood and plasma for total fat, cholesterol, and lecithin were made as follows.

The sample was drawn from the jugular vein into a 10 cc. pipette (containing a little powdered potassium oxalate) connected with the needle by means of a short length of rubber tubing, and run at once into a 15 cc. centrifuge tube also containing a little oxalate. After mixing, 3 cc. of the blood were measured with a pipette and run slowly (a slow stream of drops) into 75 cc. of a mixture of three parts alcohol and one part ether (both redistilled) in a 100 cc. graduated flask which was kept in motion during the process. The contents of the flask were raised to boiling by immersion in a boiling water bath (with constant shaking to avoid superheating), cooled to room temperature, made to volume with alcohol-ether, mixed, and filtered.

The remainder of the blood was then centrifuged for a definite time, the relative volume of corpuscles and plasma noted, and 3 cc. of the plasma were then measured out and treated in exactly the same way as the whole blood.

The alcohol-ether extracts of whole blood and plasma so obtained were sufficient for duplicate determinations of total fat, cholesterol, and lecithin which were made as follows.

Total Fat.—(Total fatty acids plus cholesterol.) For this determination 10 cc. of the extract were used. The procedure was the same as has been reported several times recently[7] and therefore need not be repeated here. The value "total fatty acids" used in the table was obtained by subtracting the value obtained for cholesterol from that of total fat.

[7] Bloor, *J. Biol. Chem.*, 1915, xxiii, 317, and earlier papers.

Cholesterol.—The method used for the determination of cholesterol has been reported recently.[8] It consists of an application of the Liebermann-Burchard color reaction as developed by Autenrieth and Funk[9] to the alcohol-ether extract of the blood and plasma. 10 cc. of the blood extract were used for the determination.

Lecithin.—The method used has been reported previously[10] but since in some of the present work a variation of the method (use of strychnine molybdate precipitation for the phosphoric acid) has been employed it is desirable to give the procedure in detail. It is as follows: 10 cc. of the extract of whole blood or 15 cc. of extract of plasma are measured with a pipette into a 200 × 25 mm. Jena test-tube, two or three glass beads of about 3 mm. diameter are added, and the solution is evaporated to dryness by immersion of the tube in a water bath. The tube should be shaken frequently until boiling has begun, after which the evaporation will proceed to dryness without further attention. The residue should be dried in the tube for 15 minutes to remove traces of alcohol which might interfere with the completeness of the subsequent oxidation. For the oxidation there are now added to the tube 1.5 cc. of a mixture of equal parts of concentrated sulfuric and nitric acids and the whole is digested by heating with a micro burner in the hood or with a Folin draught apparatus.[11] The heating is done in two stages. During the first the mixture is gently boiled with a very low flame until the red fumes have ceased to come off. This should take about 15 minutes (instead of 5 minutes as given in the original description). If the process is hastened too much at this stage the nitric acid will be driven off before oxidation is complete. The tubes should be inclined at an angle of about 30° to prevent loss by spattering. The heat is then gradually raised until the nitric acid is completely driven off, after which the solution is boiled for 8 to 10 minutes. The mixture is then cooled somewhat and two drops of 0.25 per cent cane-sugar solution are added (to destroy a nitric-phosphoric acid combination), after which the heating is resumed for

[8] Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.

[9] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

[10] Bloor, *J. Biol. Chem.*, 1915, xxii, 133.

[11] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 503.

about 1 minute. The sugar should cause a slight browning of the liquid which quickly disappears on heating. The tube is then cooled and about 3 cc. of water are added, washing down the sides of the tube.

The phosphoric acid is determined nephelometrically either by precipitation as the silver salt as in the original method or as strychnine phosphomolybdate, using Kober and Egerer's[12] modification of the Pouget-Chouchak reagent. The results by the two precipitations are practically identical. The silver precipitation was used in the first six experiments, the strychnine molybdate in the remaining one. The procedure is somewhat different for the two precipitations and is as follows.

Silver Precipitation.—To the solution in the Jena test-tube is added a drop of phenolphthalein (0.3 per cent) and the acid is exactly neutralized as follows. 20 per cent NaOH (free from chlorides and containing but little carbonate) is added to neutralization, noting the amount used, 0.5 N sulfuric acid run in till just acid, the solution cooled, and 0.1 N NaOH added until it is just alkaline again. To the neutralized solution are added 1 cc. of 10 per cent $(NH_4)_2SO_4$ and 1.5 cc. of 0.1 N NaOH and the solution is made to 10 cc. (indicated with sufficient accuracy by a scratch on the tube). A standard phosphate solution is similarly prepared as follows. 3 cc. of a phosphate solution (containing 0.15 mg. H_3PO_4) are measured into a similar Jena test-tube, a drop of phenolphthalein is added, and then the amount of 20 per cent alkali that was used for neutralization of the test solution is run in. Concentrated sulfuric acid is added till just acid, the excess of acid neutralized by a drop or two of the strong alkali, the solution cooled, and then exactly neutralized as above. 1 cc. of 10 per cent $(NH_4)_2SO_4$ and 1.5 cc. of 0.1 N NaOH are added and the solution is made to the 10 cc. mark on the tube. Two samples of 10 cc. each of 2 per cent $AgNO_3$ (neutral) are measured into 25 cc. glass stoppered graduated flasks and the standard and test solutions in the Jena test-tubes are added through a funnel with the stem drawn out so that the 10 cc. are delivered in about 15 seconds. The liquid in the flask is gently rotated while the phosphate solution is being run in, then the test-tube

[12] Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

rinsed out with water, and the wash water poured in through the funnel. Finally the liquid in the flask is brought up to the mark by rinsing the funnel with distilled water and the whole well mixed, when it is ready to read in the nephelometer.

Strychnine Molybdate Precipitation.—For this precipitation Kober's[12] strychnine molybdate reagent in HCl was used. Contrary to Kober's findings good results had been obtained nephelometrically with the reagent as originally devised by Pouget and Chouchak[13] but Kober's modification is so much of an improvement that there is no longer any reason for using the original. As was pointed out[14] for the original reagent, account must be taken of the salt content, reaction, etc., of the solutions because of the effect of these factors on the nature of the precipitate, and for that reason certain modifications in Kober's procedure are necessary. The process as applied in this work is as follows.

To the solution in the Jena test-tube is added a drop of phenolphthalein and it is then neutralized with 20 per cent NaOH as before, noting the amount added. The solution is then made just acid with a drop or two of 1 : 1 HCl, cooled, transferred quantitatively to a 25 cc. graduated flask, and made up to volume.

3 cc. of the standard phosphate solution are measured into another 25 cc. graduated flask, a drop of phenolphthalein is added, and the amount of 20 per cent NaOH used in the test solution run in. The solution is then neutralized with concentrated H_2SO_4, cooled, and made to volume. 10 cc. of each solution are used for the precipitation, which is carried out according to Kober's directions except that as a consequence of the salt content of the solution a stronger acid must be used to prevent decomposition of the reagent.

The precipitation is carried out as follows: To each of two 50 cc. graduated flasks are added about 25 cc. of distilled water, 5 cc. of 1:1 HCl, and 5 cc. of the molybdate reagent; then 10 cc. of the phosphate solutions are measured in with a pipette, keeping the flasks gently rotating during the addition. Water is added to the mark, the solutions are mixed by cautiously inverting several times, and, after standing 3 minutes, compared in the nephelometer.

The strychnine molybdate reagent possesses the advantage that while more sensitive to phosphoric acid than the silver reagent it is less sensitive to impurities. The values obtained, multiplied by eight, give a close approximation to the phosphatide values.

[13] Pouget, I., and Chouchak, D., *Bull. Soc. chim.*, series 4, 1909, v, 104
[14] Bloor, *J. Biol. Chem.*, 1915, xxii, 135.

Direct determinations were made on both whole blood and plasma from which, knowing the percentage of corpuscles in the blood the composition of the corpuscles was calculated. Direct determinations on the corpuscles by the above procedure do not appear feasible because of the apparently unavoidable clumping[15] of the corpuscles during the extraction. The error in each of the above methods as ordinarily carried out is 3 to 5 per cent.

Experiments were carried out on two different dogs as follows.

Experiment 1.—Dog 25, a female bull terrier, weight 8.35 kg., in very poor condition, was fed 50 cc. of olive oil (forced feeding)[16] at 8.55 a.m. The 1st sample of blood was taken at 9.00 a.m. After taking a 3 cc. sample for whole blood the remainder was centrifuged for 7 minutes at 2,000 R. P. M. The corpuscles were found to be 35 per cent. The plasma was slightly cloudy and yellow in color. A 3 cc. sample was taken for analysis.

		Corpuscles. per cent	Plasma.
2	11.00 a.m...............	37	Yellowish white and cloudy.
3	1.00 p.m....	37	Milky white.
4	3.00 "	35	Less milky than in 3.
5	5.00 "	37	More milky.

Experiment 2.—Dog 25 as before. Fed 60 cc. of olive oil at 8.45 a.m.

1st blood sample at 8.50 a.m., centrifuged 10 minutes at 2,000 R. P. M. Corpuscles 34 per cent. Plasma cloudy. Other samples as follows:

		Corpuscles. per cent	Plasma.
2	10.50 a.m.......................	35	Cloudy.
3	12.50 p.m.......................	35	Milky.
4	2.50 "	34	"
5	4.50 "	36	Slightly cloudy.

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[15] Later experiments have shown that the clumping may probably be avoided by more careful addition of the corpuscles to the alcohol-ether.

[16] A method of forced feeding of liquids which has been found very useful, especially for dogs, was suggested by Mr. G. L. Foster of this laboratory. The dog's head is held with one hand while with the other the lips are pulled away from the jaws on one side producing a pouch into which the liquid is poured and from which it runs into the throat and is swallowed naturally. This method does away with the necessity of a stomach tube and avoids the complications which occasionally accompany its use, especially by beginners.

Experiment 3.—Dog 25, weight 8.88 kg. (gain of 0.52 kg. since last experiment). Fed 55 cc. of olive oil at 8.45 a.m.

1st sample at 8.50 a.m., centrifuged 10 minutes at 3,800 R. P. M. Corpuscles 36 per cent. Plasma clear.

		Corpuscles. per cent	Plasma.
2	10.45 a.m.	32	White, cloudy.
3	12.45 p.m.	30	Milky.
4	2.45 "	30	White, cloudy.
5	4.45 "	30	Milky.

About 10 cc. of the oil were lost by vomiting during the first period.

This centrifuge at the above speed and time was used for the remainder of the experiments.

Experiment 4.—Dog 25 as above. Fed 55 cc. of olive oil at 8.45 a.m.

1st sample at 9.00 a.m. Corpuscles 32.5 per cent. Plasma clear. About 200 cc. of water were given at this time. Other samples as follows:

		Corpuscles. per cent	Plasma.
2	10.50 a.m.	33	White, cloudy.
3	1.00 p.m.	32.5	Milky.
4	2.45 "	Not determined.	Nearly clear.
5	4.45 "	30	Milky.

Experiment 5.—Dog 26, female collie in good condition, weight 17 kg. Fed 100 cc. of olive oil at 8.45 a.m.

1st blood sample at 8.50 a.m., centrifuged as above. Corpuscles 45 per cent. Plasma clear.

		Corpuscles. per cent	Plasma.
2	10.45 a.m.	45	Somewhat milky.
3	12.45 p.m.	44	Milky.
4	2.45 "	40	Slightly milky.
5	4.45 "	40	Milky.

Experiment 6.—Dog 26. Fasted 1 day (40 hours since last feeding). Fed 100 cc. of olive oil at 8.40 a.m.

		Corpuscles. per cent	Plasma.
1	8.45 a.m.	50	Clear.
2	10.45 "	44	White, cloudy.
3	12.45 p.m.	44	Milky.
4	2.45 "	44	Slightly white, cloudy.
5	4.40 "	44	Milky.

Experiment 7.—Dog 26.   Fed 100 cc. of olive oil at 8.40 a.m.

		Corpuscles. *per cent*	Plasma.
1	8.45 a.m....	48	Clear.
2	10.45 "	44	White, cloudy.
3	12.45 p.m.....................	44	Milky.
4	2.45 "	44	"
5	4.45 "	44 ·	Nearly clear.

In view of the recognized effect on the blood sugar, of excitement and struggling, and of a possible similar effect on blood fat, the dogs used were previously accustomed to the handling so that during the experiments they did not struggle or show other evidences of excitement. It is believed, therefore, that this factor was practically eliminated. Still an occasional high value in the first sample (as for example, Experiment 7, total fatty acids in whole blood) may have been due to this cause.[17]

Attention is called to the changes in percentage volume of the corpuscles during certain of the experiments, particularly noticeable in Experiments 3, 5, 6, and 7. The change is always a decrease, amounting in some cases to more than 15 per cent. The samples were centrifuged in the same centrifuge and for the same periods of time and at the same speed. Tests of the speed of the centrifuge from time to time on different days and times of the day showed that it did not change notably. Also in the experiments noted above no water was given so that there can be no question of dilution of the blood from that source. These changes in percentage of the corpuscles are in all probability due to increases in plasma volume as a result of the increased flow of lymph into the blood by way of the thoracic duct during fat absorption. The varying figures show that the volume of the blood cannot be assumed to remain constant during experiments of this kind.

The analytical results of the experiments are given in Table I below.

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[17] This factor may also have been responsible for the lower and more irregular results obtained in the previous experiments.

TABLE I.

The Blood Lipoids during Fat Absorption:
Gm. per 100 cc.

No. of experiment	Time.	Total fatty acids.			Lecithin.			Cholesterol.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
1	Before..	0.52	0.57	0.43	0.32	0.30	0.36	0.20	0.15	0.31
	2 hrs....	0.63	0.67	0.56	0.38	0.30	0.51	0.20	0.16	0.28
	4 " ...	0.97	0.92	1.05	0.54	0.34	0.88	0.22	0.18	0.31
	6 " ...	0.68	0.58	0.85	0.47	0.32	0.72	0.22	0.19	0.30
	8 " ...	0.71	0.75	0.64	0.40	0.32	0.52	0.22	0.19	0.30
2	Before..	0.63	0.60	0.69	0.35	0.29	0.47	0.23	0.20	0.29
	2 hrs....	0.69	0.60	0.86	0.41	0.30	0.62	0.23	0.20	0.29
	4 " ...	0.72	0.61	0.92	0.48	0.30	0.85	0.22	0.19	0.28
	6 " ...	0.77	0.68	0.94	0.48	0.30	0.83	0.21	0.19	0.28
	8 " ...	0.76	0.64	0.70	0.34	0.30	0.44	0.21	0.21	0.28
3	Before..	0.53	0.54	0.50	0.32	0.28	0.41	0.18	0.16	0.22
	2 hrs....	0.59	0.59	0.59	0.36	0.28	0.53	0.18	0.16	0.22
	4 " ...	0.71	0.69	0.76	0.41	0.30	0.67	0.19	0.16	0.26
	6 " ...	0.58	0.61	0.54	0.42	0.30	0.65	0.19	0.16	0.26
	8 " ...	0.61	0.69	0.46	0.37	0.31	0.49	0.19	0.16	0.26
4	Before..	0.53	0.57	0.45	0.32	0.33	0.31	0.19	0.15	0.28
	2 hrs....	0.57	0.59	0.53	0.34	0.35	0.32	0.17	0.15	0.21
	4 " ...	0.75	0.72	0.81	0.41	0.37	0.50	0.17	0.15	0.21
	6 " ...	0.75	0.72	0.81	0.36	0.30	0.47	0.17	0.16	0.19
	8 " ...	0.70	0.62	0.87	0.40	0.28	0.65	0.18	0.16	0.25
5	Before..	0.63	0.70	0.65	0.32	0.30	0.31	0.30	0.31	0.29
	2 hrs....	0.76	0.79	0.73	0.37	0.34	0.40	0.29	0.31	0.27
	4 " ...	0.93	0.99	0.84	0.41	0.34	0.50	0.28	0.31	0.24
	6 " ...	0.83	0.86	0.81	0.36	0.36	0.36	0.30	0.30	0.30
	8 " ...	0.90	0.93	0.86	0.36	0.33	0.40	0.28	0.31	0.24
6	Before..	0.68	0.76	0.60	0.40	0.50	0.30	0.30	0.37	0.23
	2 hrs....	0.80	0.91	0.66	0.47	0.53	0.40	0.29	0.36	0.20
	4 " ...	1.08	1.15	1.00	0.53	0.51	0.56	0.28	0.34	0.20
	6 " ...	0.89	0.90	0.88	0.46	0.52	0.40	0.29	0.35	0.21
	8 " ...	0.95	0.96	0.94	0.48	0.51	0.44	0.28	0.33	0.22
7	Before..	0.64	0.62	0.66	0.40	0.44	0.36	0.21	0.23	0.19
	2 hrs....	0.62	0.56	0.70	0.41	0.44	0.38	0.23	0.25	0.19
	4 " ...	0.90	0.95	0.84	0.49	0.43	0.56	0.23	0.25	0.19
	6 " ...	0.73	0.86	0.75	0.42	0.39	0.46	0.23	0.24	0.22
	8 " ...	0.81	0.73	0.91	0.42	0 42	0.42	0.23	0.24	0.22

DISCUSSION.

Total Fatty Acids.—(Combined in various forms—glycerides, lecithin, cholesterol esters—and free, as soaps or fatty acids.) The increases in whole blood and plasma are those long known to take place in the blood during fat feeding (alimentary lipemia). The greatest increase noted in the above experiments was 86 per cent for whole blood and 61 per cent for plasma. More striking, however, are the increases in total fatty acids in the corpuscles. With two exceptions (Experiments 5 and 7) these are much greater than in either whole blood or plasma. In Experiment 1 the greatest increase in whole blood is 86 per cent, plasma 61 per cent, corpuscles 144 per cent; in Experiment 4, the increases are, whole blood 42 per cent, plasma 27 per cent, corpuscles 86 per cent. These results bear out the findings of Munk[1] who in 1901 reported that the fat of the corpuscles increased during fat absorption and indicate that the corpuscles actively absorb the fat from the plasma.

Another point shown in most of the above experiments and referable probably to the way in which the liquid fats pass from the stomach is the decrease in the fat of whole blood and plasma at the fourth period (6th hour) after feeding and the increase again at the fifth period. Apparently the larger quantity of oil passes at once to the intestine like other liquids and the remainder not till a later period (when the hunger contractions begin?).

Cholesterol (Total).—The changes in cholesterol are slight and inconstant, thus bearing out the findings of the earlier experiments, and confirming the probability that cholesterol takes no part in the early stages of fat metabolism.

Lecithin (Phosphatides).—In all these experiments as in the earlier ones, lecithin is found to increase in the blood during fat absorption.

In the whole blood the greatest increase noted is in Experiment 1, where the lecithin increases 70 per cent. In the other experiments the increases are all about 30 per cent. The changes in the plasma are much less striking than in the whole blood, varying from 13 per cent in Experiment 1, to none in Experiment 7, and averaging about 10 per cent. The increases in the corpuscles are, on the other hand, most marked, varying from 144 per cent

in Experiment 1 to 55 per cent in Experiment 7, and averaging 83 per cent. These great increases together with the similar increases of the total fatty acids in the corpuscles noted above, are believed to be conclusive evidence that the blood cells absorb the fat from the plasma and transform it into lecithin. Quite in agreement with the above as establishing the relation between the blood cells and lecithin is the recent work of Thiele[18] and of Foà[19] who report the presence in blood of an esterase which decomposes the lecithin of the blood on standing, a change which takes place only in the presence of the corpuscles. Since these enzyme reactions in the living organism are generally regarded as reversible, the *formation* of lecithin in the presence of excess of fat in the blood as above is to be expected. Also, if the reaction were a reversible one, the relation between the fat and the lecithin would be expected to approximate a constant (as indeed has been reported for the blood corpuscles by Mayer and Schaeffer.[20] On the other hand, in view of the constantly changing conditions prevailing in the blood during fat absorption and the limitations of the methods employed for the analyses, it was hardly to be expected that an exactly constant relation between the total fatty acids and the lecithin could be demonstrated during the fat absorption, but there is a rather surprising degree of constancy in most cases as may be seen from Table II.

TABLE II.

Relationship between Total Fatty Acids and Lecithin $\left(\dfrac{T.F.A.}{L}\right)$ *in Whole Blood and Corpuscles.*

No. of experiment.	1		2		3		4		5		6		7	
Period.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.
1	1.6	1.2	1.8	1.4	1.7	1.2	1.7	1.6	1.8	2.1	1.7	2.0	1.6	1.8
2	1.7	1.1	1.7	1.4	1.65	1.1	1.7	1.7	2.0	1.8	1.7	1.7	1.5	1.8
3	1.8	1.2	1.5	1.1	1.7	1.1	1.8	1.6	2.1	1.7	2.0	1.8	1.8	1.5
4	1.5	1.2	1.6	1.1	1.4	0.9	2.1	1.7	2.1	2.3	1.9	2.2	1.8	1.6
5	1.7	1.2	2.3	1.6	1.65	1.0	1.8	1.4	2.4	2	2.0	2.1	2.1	2.2

[18] Thiele, F. H., *Biochem. J.*, 1913, vii, 275.

[19] Foà, C., *Arch. ital. biol.*, 1915, lxiii, 239.

[20] Mayer, A., and Schaeffer, G., *J. physiol. et path. gén.*, 1913. xv, 984.

The constancy in the relationship between the fat and the lecithin may also be taken as evidence that all the absorbed fat passes through the lecithin stage and that therefore lecithin is to be regarded as the first stage in fat metabolism.

The hitherto unexplainable anatomical mechanism (thoracic duct) by which the fats are thrown directly into the blood stream and thoroughly mixed with the blood in the heart and lungs before they reach any of the organs of intermediate metabolism (e.g., liver) may be explained on the basis of the above results. Since the first step in the metabolism of the fats takes place in the blood corpuscles it is to be expected that they be given an opportunity to take up as much of the fat as possible before it comes in contact with the organs and tissues.

As to which of the blood cells—red or white—take the predominating part in the lecithin formation, there is no direct information. It has long been known that during fat absorption the white blood cells in the intestinal lymph spaces become loaded with fat and apparently take an active part in the absorption, so that the idea would occur to many that the white cells were responsible for the lecithin increase in the blood. However in view of the fact that they constitute less than 0.5 per cent of the total blood cells it would seem to be physically impossible for them to transform the relatively great amount of fat taken into the blood during a fat meal.

It is quite unlikely, on the other hand, that the lecithin formation is limited to the blood cells. Esterases have been found in many tissues,[21] and it is probable that the circulating fat is removed from the blood stream and worked up by many types of fixed cell in the same way as it is done by the blood cells.

SUMMARY.

Determinations of total fat, lecithin (phosphatides), and cholesterol have been made in whole blood and plasma (and by calculation in the corpuscles) during a series of fat absorption experiments, with the following results.

1. Total fatty acids increase in both plasma and corpuscles but the increase is generally more marked in the corpuscles.

[21] Loevenhart, A. S., Am. J. Physiol., 1901–02, vi, 331.

2. Lecithin increases greatly in the corpuscles but only slightly in the plasma.

3. No definite changes in the quantity of cholesterol were noted.

4. A fairly constant relationship between total fatty acids and lecithin was noted in whole blood and corpuscles.

These results are believed to justify the conclusions: (a) that the blood corpuscles take up the fat from the plasma and transform it into lecithin; (b) that most if not all of the absorbed fat is so transformed; and therefore (c) that lecithin is an intermediate step in the metabolism of the fats.

THE ORIGIN OF THE NITRATES IN THE URINE.

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Nitrates appear to be constant constituents of human urine. Calculated as the potassium salt, human urine contains[1] ordinarily 100 to 200 mg. of nitrate per liter, and occasionally 300 to 400 mg. or more. On normal rations other animals also excrete nitrates in the urine. The question of the origin of the urinary nitrates has not been settled satisfactorily, at least in the case of human urine. Thus the evidence is not conclusive as to whether they are derived only from the food or whether they are formed to some extent by animal tissues. The problem is of peculiar theoretical interest, since the production of an oxidized nitrogenous radicle by animal tissues would be unique. A somewhat extended historical introduction to this rather obscure question seems necessary for a proper appreciation of the experiments described later.

In the oxidation of nitrogenous organic compounds containing nitrogen in amino or imino groupings, one of the first steps in the process seems to be a splitting off of the nitrogen, either directly, as in the deamination of amino-acids and amino purines, or after a hydrolytic cleavage, as in the utilization of polypeptides or tissue protein. The nitrogen thus removed subsequently appears in the urine largely as urea, ammonium salts, or, in the case of birds and reptiles, as uric acid. In the case of amino aromatic bodies, in which the amino group is substituted in the carbocyclic nucleus, the amino group undergoes no change in the animal organism if the nucleus itself remains intact, aside from conversions into acetyl, uramido, or methyl substituted groups. Again, nitrile or cyanide radicles are eliminated from the body as inorganic sulfocyanides.[2]

From such facts as the above, it is evident that the body is unable to derive energy from the nitrogen atom to any great extent, at least, by

[1] Caron, H., *Ann. chim. anal.*, 1912, xvii, 9.

[2] Lang, S., *Arch. exp. Path. u. Pharm.*, 1895, xxxvi, 75.

combining it with oxygen, in marked contrast to the ease with which it oxidizes the carbon and hydrogen atoms. In fact, the organism has a strong reducing action towards nitrogen in an oxidized condition. Thus, nitrobenzene taken *per os* is excreted in the urine in part as *p*-aminophenol, while *o*-nitrophenol appears to be excreted unchanged.[3] Phenylhydroxyla-mine, as well as its nitroso derivative, has recently been shown by Sie-burg[4] to be in part, at least, transformed into *p*-aminophenol and to be excreted in the urine as the lactam of *p*-aminophenol glucuronic acid.

An interesting illustration of the reduction of radicles containing nitro-gen in an oxidized condition, is the fate of *m*-nitrobenzaldehyde in the animal organism. Cohn[5] has shown that this compound, when introduced into the rabbit, suffers oxidation of the aldehyde to the carboxyl group, while simultaneously the nitro group is reduced to the amino group, which is acetylated, so that the final product is *m*-acetylaminobenzoic acid. In the body of the dog, however, this transformation does not take place, the final product in this case being urea *m*-nitrohippurate.

Ingested inorganic nitrates are known to be partially and in some cases almost entirely reduced. Thus, Röhmann[6] after giving rabbits 30 to 50 mg. of potassium nitrate in the food was only able to recover 54 to 70 per cent in the urine. Of 500 mg. of potassium nitrate subcutaneously injected, only 55 per cent was excreted in the urine. With dogs even smaller percentages than these were recovered in the urine. Grüter[7] was unable to recover any portion of nitrate doses from the urine of a rabbit either when given *per os* or intravenously. His method of determining nitrates in urine was, however, open to objection. In two experiments on the intravenous injection of nitrates into rabbits, Neuffer[8] was able to recover only 41 per cent in the urine. As was also the case in Röhmann's work, all of the nitrate was not eliminated from the body until 4 or 5 days after the nitrate was given.

Such observations as the above on the destruction of inorganic nitrates by the body have been repeatedly confirmed, though the results are remark-ably variable as to the extent of reduction. Thus, Weyl and Citron,[9] in experiments with a dog, were able to recover only 12 per cent of the ingested nitrate in the urine, and with ducks and hens only about 33 per cent; while

[3] Meyer, E., *Z. physiol. Chem.*, 1906, xlvi, 497.

[4] Sieburg, E., *Z. physiol. Chem.*, 1914, xcii, 331.

[5] Cohn, R., *Z. physiol. Chem.*, 1893, xvii, 274; *Arch. exp. Path. u. Pharm.*, 1905, liii, 435.

[6] Röhmann, F., *Z. physiol. Chem.*, 1881, v, 233.

[7] Grüter, L., Über das Schicksal der salpetersauren Salze im Tierkörper, Dissertation, Würzburg, 1895.

[8] Neuffer, L., Ueber das Verschwinden von Salpetersäure im Stoffwechsel. Dissertation, Würzburg, 1898.

[9] Weyl, T., and Citron, *Arch. path. Anat. u. Physiol.*, 1885, ci, 175. Weyl, T., *ibid.*, 1886, cv, 187.

Gossels[10] was unable to detect any increase in the nitrates of human urine, even after the ingestion of as much as 3 gm. of potassium nitrate. On the other hand, recent work by Grafe and Wintz,[11] and Abderhalden and Hirsch,[12] on dogs and pigs indicates that ingested nitrates may be quantitatively eliminated in the urine in some cases. The greatest destruction of ingested nitrate in these experiments amounted only to 10 or 15 per cent. These discrepancies are probably in large part due to different methods used in the determination of nitrates in the urine, and in part to the use of different species of experimental animals; but after allowing for these factors it seems probable that the extent of the destruction of nitrates taken with the food or injected directly into the blood may vary within wide limits.

The location of the reduction of nitrates in the body has been the subject of many researches.[13] In the course of many investigations on the action of bacteria on nitrates, a subject of great importance to agriculture, it has been shown that many of the bacteria occurring in the intestines of animals are capable of reducing nitrates to nitrites. Thus, *B. coli communis* has the power of reducing nitrates to nitrites,[14] while the occasional production of the symptoms of nitrite poisoning after nitrate ingestion,[15] or after the injection of bismuth subnitrate into the intestine for radiographic investigations,[16] points to the capacity of the intestinal contents of accomplishing the first step in the reduction of nitrates.

That the reducing action of intestinal bacteria on nitrates does not stop at this stage, but may continue to the formation of ammonia and nitrogen gas has also been determined. Thus, Maassen found that, especially in the presence of carbohydrates or polyatomic alcohols, *B. coli communis* was able to destroy nitrates and nitrites entirely in artificial culture media in the course of 2 weeks. Burri and Stutzer[17] showed that horse feces pos-

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[10] Gossels, W., Die Nitrate des Tier- und Pflanzenkörpers, Dissertation, Berlin, 1886.

[11] Grafe, E., and Wintz, H., *Z. physiol. Chem.*, 1913, lxxxvi, 283.

[12] Abderhalden, E., and Hirsch, P., *Z. physiol. Chem.*, 1913, lxxxiv, 189.

[13] In the preparation of this review of the literature, particularly of that portion immediately following, we wish to acknowledge the assistance obtained from an unpublished manuscript of Professor A. P. Mathews on "The Pharmacology of Nitrates and Nitrites."

[14] Maassen, A., *Arb. k. Gsndhtsamte.*, 1902, xviii, 21. Pelz, E., *Centr. Bakt., 1te Abt., Orig.*, 1910, lvii, 1.

[15] Barth, A. F., Toxicologische Untersuchungen über Chilisalpeter. Inaug. Dissertation, Bonn, 1879. Binz, C., and Gerlinger, P., *Arch. int. Pharm. et Thérap.*, 1901, ix, 441.

[16] Böhme, A., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 441. Rautenberg, E., *Berl. klin. Woch.*, 1906, xliii, 1397. Worden, C. B., Sailer, J., Pancoast, H. K., and Davis, G. G., *Univ. Pennsylvania Med. Bull.*, 1906. Zadek, J., *Z. exp. Path. u. Therap.*, 1914, xv, 498.

[17] Burri, R., and Stutzer, A., *Centr. Bakt., 2te Abt.*, 1895, i, 257.

sessed the property of developing free nitrogen from nitrates, a fact which has been confirmed by Grüter.[7,18] The former investigators isolated from manure a denitrifying bacillus which in symbiosis with *B. coli communis* was able to liberate nitrogen gas from nitrate solutions. These denitrifying bacilli seem to be more active in the feces from the herbivora, though Maassen has frequently obtained such bacteria from human feces.

The presence in the intestinal contents, especially of the large intestine, of nitrite-forming and denitrifying bacteria and also the production of nascent hydrogen, which is constantly taking place in the intestines, might well account for the partial destruction of nitrates taken *per os.* It would, however, have no bearing on the partial destruction of nitrates injected directly into the body, subcutaneously or intravenously, especially since it has been shown by Neuffer that in such experiments excretion of nitrates into the intestine with subsequent reduction does not occur. Evidently in such cases, the tissues themselves must exert a reducing action. That the tissues possess strong reducing powers, illustrated by the formation of fats from carbohydrates, the production of hydroxy-acids from keto-acids, the reduction of arsenic to arsenious acid, of the nitro group to the amino group, of substituted hydroxylamines to amino compounds, of iodates to iodides, chlorates to chlorides, and sulfur to hydrogen sulfide, is well established. Similarly, the capacity of organ extracts to reduce nitrates, at least to nitrites, seems fairly well established. Gscheidlen[19] obtained some evidence that the muscle tissue of a frog was able to reduce nitrates to nitrites, and that active muscles were more powerful in this respect than inactive. Mulzer,[20] however, was unable to confirm this result, although he was able to detect a strong reducing action on nitrates by many of the internal organs. Neuffer[8] incubated various tissues and organs of a rabbit for 24 hours with definite amounts of a 1 per cent solution of sodium nitrate and was able to demonstrate a loss of nitrate, especially in the case of liver tissue, and to some extent in the case of muscle tissue and blood. Boiled liver tissue had no demonstrable effect.

The results of Neuffer have been confirmed by Abelous and Gérard.[21] These investigators incubated macerated horse kidney with a solution of potassium nitrate. After 12 to 15 hours the presence of nitrites could be demonstrated by several reactions. The reactions were not obtained if the kidney pulp was previously heated to 100° C. It was found that the reducing substance could be extracted by water or glycerol and that its activity varied with the temperature in a similar manner to enzyme action. Other tissues were tested in a similar manner and were found to have a

[18] Zuntz, H., *Arch. Anat. u. Physiol., Physiol. Abt.,* 1886, 560.

[19] Gscheidlen, R., *Arch. ges. Physiol.,* 1874, viii, 506.

[20] Mulzer, M., Toxicologische Studien über das Natriumnitrat mit Beziehung auf andere Natriumsalze, Inaug. Dissertation, Würzburg, 1892; *Jahresb. Tierchem.,* 1900, xxx, 101.

[21] Abelous, E., and Gérard, E., *Compt. rend. Acad.,* 1899, cxxix, 56, 164, 1023.

similar action, though in very unequal degree. The liver, kidney, and suprarenal tissues seemed particularly powerful, while muscle and brain tissues were relatively weak in their action. In all their work, bacterial action was excluded by the use of either chloroform, sodium fluoride, or thymol. The results of Abelous and Gérard were later confirmed by Vogelsohn,[22] who also concluded that extracts of liver and kidney tissue contain enzymes capable of converting nitrates into nitrites. In this work, a large part of the nitrite formed had apparently been further reduced, so that it escaped recognition.

In view of the investigations just mentioned, it seems probable that the tissues are able to reduce nitrates to nitrites and possibly to ammonia, though it is evident that under average conditions the nitrite stage must be transient, because the entrance into the adult body, either enterally or parenterally, of considerable amounts of nitrate is only occasionally followed by symptoms of nitrite poisoning. Such symptoms would be produced by very minute amounts of nitrite, the poisonous action of nitrites being extremely potent. The subsequent steps in the reduction of nitrates beyond the nitrite stage is conjectural. Franzen and Löhmann[23] consider exhaustively the possibility of a conversion into ammonia through dihydroxy ammonia and hydroxylamine. It seems probable that ammonia would finally be produced by the tissues rather than free nitrogen, as suggested by Röhmann, though no experimental evidence exists either one way or the other.

While undoubtedly the preponderating tendency in animal metabolism is towards the production and subsequent excretion of unoxidized nitrogen, there is good evidence that under certain conditions nitrogenous radicles may be transformed from a less to a greater oxidized condition. The best evidence on this point concerns the conversion of nitrites to nitrates. Under normal conditions the evidence,[6,24] indicates that nitrites are not excreted in the urine, while nitrates are. Apparently nitrites formed in metabolism from the reduction of nitrates ingested with the food are further reduced in the tissues. Also, when moderate amounts of inorganic nitrates are added to the food or subcutaneously injected into an animal, the fresh urine is free from nitrites. However, from the work of Barth and later of Binz and Gerlinger,[15] it appears that when excessive amounts of inorganic nitrates are given to an animal, the fresh urine may respond to nitrite tests and may contain even considerable amounts of nitrite.

[22] Vogelsohn, A., Ueber die Einwirkung von Organextrakten auf Nitrate und Nitrite, Inaug. Dissertation, Bern, 1907; *Jahresb. Tierchem.*, 1908, xxxviii, 874.   Schönbein, C. F., *Z. Biol.*, 1867, iii, 334.   Stepanow, A., *Arch. exp. Path. u. Pharm.*, 1902, xlvii, 411.   Thunberg, T., *Ergebn. Physiol.*, 1911, xi, 328.

[23] Franzen, H., and Löhmann, E., *Z. physiol. Chem.*, 1909, lxiii, 100. Neuberg, C., and Welde, E., *Biochem. Z.*, 1914, lxvii, 18.

[24] Schönbein, *J. prakt. Chem.*, 1864, xciii, 463.

The excellent work of Röhmann in 1881 proved that even after subcutaneous injection of moderate amounts of sodium nitrite into rabbits the fresh urine of the same and subsequent days failed to respond to the nitrite test. On the other hand, after the injection of 100 to 500 mg. of this salt, the urine, which previously was nitrate-free,[25] was found to contain nitrates for 2 or 3 days following the nitrite injection. However, only 36 to 43 per cent of the quantity of nitrite injected could be accounted for by the quantity of nitrate appearing in the urine, the remainder apparently suffering reduction in the body.

These observations of Röhmann have been confirmed by Mulzer[26] and Atkinson.[26] Mulzer, after injecting rabbits with sodium nitrite, was unable to detect nitrites in the fresh urine. On standing, however, a nitrite reaction developed, obviously from preformed nitrates. Atkinson administered sodium nitrite to rabbits, and very rarely found nitrites in the urine, even after fatal doses had been given. Nitrates, however, were always present, though the animal was on a diet which produced no excretion of nitrates in the urine. In agreement with Röhmann, Atkinson was able to recover only a part of the administered nitrite as nitrate—in the one experiment cited, 47 per cent. In no case were nitrates or nitrites found in the feces. In the case of dogs, nitrites could readily be detected in the urine for 24 to 36 hours after the subcutaneous injection of 130 to 194 mg. of sodium nitrite. The more vigorous the dog was, the less readily was the nitrite detected. The same circumstance was found to hold in the case of man, men in vigorous health rarely having nitrite reactions in their urine, even after considerable doses, while patients in bed give it comparatively readily.

Other instances of the oxidation of nitrogen have been reported, though they have never been confirmed. We refer to the early experiments of Bence-Jones[27] on the oxidation of ammonium salts to nitric acid. This investigator seems to have obtained clear-cut evidence with human subjects in support of his conclusions, but Wulffius,[28] using Bence-Jones' analytical methods with a man as subject, and Weyl and Citron,[9] experimenting on dogs, were unable to confirm them. Ville and Mestrezat,[29] in their report of investigations on the origin of the nitrites of the saliva, state that pure parotid and submaxillary saliva contains nitrates, which are subsequently reduced to nitrites in the mouth cavity. They also state that the nitrate content of saliva may be increased by the ingestion of capsules of sodium nitrate, sodium nitrite, or ammonium salts. No 'details of the experimental technique or of any quantitative results are

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[25] On a ration of milk and wheat bread.

[26] Atkinson, G. A., *J. Anat. and Physiol.*, 1887–88, xxii, 351.

[27] Bence-Jones, H., *Phil. Tr. Roy. Soc. London*, 1851, cxli, 399.

[28] Wulffius, E., Ueber den Nachweis von Salpetersäure im Harn, Inaug. Dissertation, Dorpat, 1861.

[29] Ville, J., and Mestrezat, W., *Compt. rend. Soc. biol.*, 1907, lxiii, 231.

reported. While the production of nitrates from nitrites by the salivary glands is in line with the work of Röhmann, Mulzer, and Atkinson, the question of the oxidation of ammonium salts to nitrates is in need of further confirmation.

Recently Mazé[30] claims to have proven the production of nitrous acid by living animal cells. Blood corpuscles of a rabbit were separated aseptically from the plasma by centrifugation, washed three times in physiological (0.9 per cent) salt solution, and finally suspended in this solution so as to give an emulsion of the form elements of blood five to ten times more dilute than blood itself. In this medium the corpuscles remained alive for several days. If hemolysis was not too rapid, Mazé was able to detect nitrous acid by the iodide test in less than 48 hours at ordinary temperatures and in 3 to 4 days at 10°. The reaction was neither constant nor regular, apparently depending on unknown factors. The reaction disappeared as soon as hemolysis was well under way. Mazé expresses the opinion from the results of this and other experiments on bacteria and plant juices that it is probable that nitrous acid exists in the cell in combination. The report of this work may be criticized for failure to specify all experimental details, such as the precautions taken to exclude bacterial action. It is also unfortunate that some more specific tests than the iodide test were not employed for the detection of nitrous acid, while the inconstant and variable results obtained cannot but detract from the confidence in the particular interpretation given to them by Mazé. This investigator also claims to have proved the presence of nitrous acid in fresh urine under normal conditions.

The possibility that the body is able to oxidize ammonium salts or amino bodies to nitrates has been tested by several investigations on the origin of nitrates in the urine. Nitrates constantly occur in the urine of animals on normal rations. The wide distribution of nitrates in food materials, especially in fresh vegetables,[31] has led to the common supposition, expressed in text-books on physiological chemistry, that the urinary nitrates have their sole origin in the food consumed. There is good evidence substantiating this view. Röhmann was unable to detect nitrates in the urine of dogs fed exclusively on meat or in the urine of rabbits fed exclusively on milk and wheat bread. The work of Röhmann seems reliable and is extensively quoted. It was later completely confirmed by Weyl and Citron.[32]

On the other hand, the results with human subjects seem to bear another

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[30] Mazé, *Compt. rend. Acad.*, 1911, clii, 1624; 1911, cliii, 357. In his second paper Mazé mentions a confirmation of Bence-Jones' work by Müntz, in the nature of a verbal communication. An exhaustive review of the literature, however, has failed to reveal any published report of such work.

[31] Richardson, W. D., *J. Am. Chem. Soc.*, 1907, xxix, 1757.

[32] Weyl and Citron, *Arch. path. Anat. u. Physiol.*, 1885, ci, 178.

interpretation. Thus, Wulffius,[28] experimenting on himself, eliminated all vegetables from his diet and used distilled water in the preparation of all food except of the bread used. Upon distilling the concentrated urine with sulfuric acid and testing the distillate with the iodide method for nitrous acid, it was found that as the experiment progressed the above reaction became less and less marked, but did not disappear. Weyl and Citron[33] found that men on an exclusive meat diet still excreted nitrates in the urine, equivalent to about 35 mg. of N_2O_5 per liter. The details of these experiments are not reported. These investigators report an analysis on the urine of a patient with progressive paralysis and beginning decubitus. The patient received only milk, meat broth, and eggs, foods practically nitrate-free. The urine was found to contain about 63 mg. of N_2O_5 per liter. Gossels[10] in Weyl's laboratory, on a vegetable-free diet excreted from 5 to 8 mg. of N_2O_5 per day. Th : evidence cited seems to indicate that with man the excretion of nitrates in the urine is not all derived from nitrates ingested with the food. It at least furnishes the basis of a fair suspicion that the nitrate metabolism of man may be qualitatively different from that of the dog and the rabbit.

Some experimental results obtained in this laboratory in 1907–08 on men afford strong support to the view that the human organism is able to produce nitrates from unoxidized nitrogen. In the course of an extensive metabolism experiment on 24 men, the results of which will be published in detail elsewhere, the following facts as regards the metabolism of nitrates were determined.

1. For a period of 220 days, twelve of the subjects received a diet consisting largely of foods containing no nitrates or only mere traces. While the nitrate analyses on the different foods used were not as complete as might be desired, such as they were they would indicate that the nitrate intake per man per day was probably less than the equivalent of 25 mg. of potassium nitrate. During the 220 days on this diet, the 4 day composite collections of urines were occasionally analyzed for nitrates by the Schulze method, over 180 analyses being made on the urines of the twelve men. The average daily excretion was about 82 mg. of potassium nitrate. Marked individual differences were noted. Two men consistently excreted from 100 to 150 mg. per day. Two others on the same diet excreted only 30 to 35 mg. per day. However, from the data at hand it appeared highly probable that most of the twelve men were consistently excreting more nitrate than they

[33] Weyl and Citron, Arch. path. Anat. u. Physiol., 1885, ci, 187.

were ingesting, and in many cases from three to six times the amount ingested.

2. Twelve other men during a fore-period of 92 days, while consuming the same diet as the first group of men, excreted quantities of nitrate comparable to the excretions noted above. During an immediately following period of 96 days they received the same diet as in the fore-period with the addition of variable amounts of potassium nitrate, from 80 to 124 mg. per day. The increased nitrate intakes in all cases increased the nitrate excretions in the urine. With the one exception noted in the following paragraph, the increases in the daily excretion of urinary nitrates were less than the increases in the daily intake of nitrates, indicating a partial destruction of the nitrate added to the food. An interesting relation was discovered between the nitrate content of the urine and the increase in the nitrate content on the addition of nitrates to the food consumed, such that, in general, the greater the former, the greater the latter. This would indicate that the differences among the subjects as regards the quantity of nitrates excreted per day in a measure indicated differences in the ability to destroy ingested nitrates, and probably also differences in the intensity of nitrate production in the body.

3. The exceptional behavior of two of the subjects of the latter group of twelve men is worthy of special mention. One subject on the nitrate-poor diet of the fore-period excreted an average of 267 mg. of nitrate (expressed as KNO_3) in the urine per day, this figure being an average of seven analyses. During a subsequent period of 72 days, when 93 mg. of KNO_3 were added to his food per day, his urinary excretion averaged 368 mg. per day (sixteen analyses). During the next 56 days, 124 mg. of KNO_3 were given per day. His urinary excretion rose from an average of 368 mg. to an average of 490 mg. per day (thirteen analyses). From such data there seems to be little doubt that this man was producing nitrates in the body and that apparently the introduction of nitrate with the food stimulated the production of nitrate in the body. In marked contrast to this subject, a second subject, throughout the 128 days during which nitrate was added to the diet, excreted less nitrate in the urine than the amount of nitrate added. The quantity of nitrate excreted per

day in the urine averaged only 53 per cent of the quantity of
nitrate added per day to the food. The first subject averaged
about 78 kg. in weight, and the second about 57 kg. However,
considering all the subjects of the experiment, no close correla-
tion apparently existed between body weight and the excretion
of nitrates in the urine, though in general the men of large body
weight excreted the largest quantities of nitrate, while the men
excreting the smallest quantities of nitrate were in general below
the average in weight.

4. At no time were nitrates or nitrites discovered in the feces,
nor were nitrites ever detected in the fresh urines.

The results of this experiment can apparently bear only one
interpretation; i.e., that the nitrates in human urine are not
derived entirely from the nitrates in the food, the presumption
being that the body is able to form nitrates from unoxidized
nitrogenous radicles. However, there is one weak point in the
evidence, and that is that the nitrate intake was not determined
consistently or completely throughout the experiment. The foods
were analyzed occasionally in the condition in which they were
served, generally with negative results. It was therefore con-
sidered advisable to undertake experiments in which the nitrate
intake would be reduced to as low a level as practicable and in
which the nitrate balance would be accurately determined. These
experiments will now be considered.

Methods.

In all of our work we used a modification of the Schulze
method,[34] in which the nitrates are reduced to nitric oxide by
ferrous chloride and hydrochloric acid, the nitric oxide evolved
is collected over concentrated alkali, and its volume measured.
The only important modifications we have made in this method
may be summarized as follows.

1. The decomposition of the nitrates was effected in a 100 cc
Jena round bottom flask, which was heated in an oil bath
kept at a temperature of about 155°C. This method of heating

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[34] For a good description of the original method see Tiemann-Gärtner'
Handbuch der Untersuchung des Wasser, etc., Braunschweig, 4th edition
1895, 154, 176.

insures a steady decomposition, and the operation conducted in this way does not require as much attention as when a free flame is used.

2. The air was removed from the apparatus by 10 or 15 minutes' boiling of the reagents,[35] after which the solution to be analyzed was introduced gradually into the flask from a glass separatory funnel. In the Schulze method the unknown solution is boiled in the decomposition flask until the air is removed, after which the reagents are introduced. We have adopted the former procedure, since the air may be more quickly expelled by boiling the reagents, while the final results are equally reliable.

3. In determining the amount of nitric oxide in the gas collected its volume was measured before and after absorption with an alkaline permanganate solution, according to the procedure of Van Slyke in removing the nitric oxide from the nitrogen obtained in his method for the determination of aliphatic amino groups.[36] This solution contains 50 gm. of potassium permanganate and 25 gm. of potassium hydroxide per liter. In carrying out the absorption, the gas measuring tube in which the NO was collected is connected, while its lower end is still immersed in the 20 per cent caustic alkali used in our work, with the gas burette of the Van Slyke apparatus by a glass capillary tube and rubber tubing. The Van Slyke burette, the leveling bulb connected with it, and the tubes connecting the burette with the gas tube are filled with a 7 per cent[37] solution of Greenbank's alkali. By opening the stop-cock at the upper end of the gas tube and that giving admission to the gas burette, and by lowering the leveling bulb, the gas is drawn over into the burette. The burette stop-cock is closed as soon as the gas is over so as to prevent unnecessary mixing of the two alkali solutions. After standing in contact with the 7 per cent alkali for 10 minutes, the

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[35] The reagents consisted of 15 cc. of a solution of ferrous chloride (prepared by dissolving 500 gm. of clean iron nails in 3 liters of concentrated chemically pure HCl and filtering through sand over glass wool) and 10 cc. of HCl (sp. gr. 1.10).

[36] Van Slyke, D. D., J. Biol. Chem., 1911, ix, 186.

[37] This strength alkali was chosen simply for convenience in calculating the water vapor pressure corrections. The only table readily available for calculating this correction was one in Biedermann's Chemiker-Kalendar for 1914 for alkali of 7 per cent strength.

gas is measured by proper adjustment of the leveling bulb, and is then forced into the Hempel pipette containing the alkaline permanganate solution. The pipette is then shaken by a small electric motor for about 5 minutes and the residue drawn over into the burette and its volume measured. The absorption is then repeated to determine whether it is complete. The difference in volume between the original measurement and the final measurement of the residual gas not absorbed by the alkaline permanganate solution, is a measure of the nitric oxide obtained in the analysis.

4. In analyzing the foods, it was generally found advisable, especially with the starchy foods, to extract them thoroughly with 75 per cent alcohol, checking the completeness of the extraction with the diphenylamine reaction. In some cases a water extract was made. The extract was then made alkaline with sodium hydroxide and evaporated on the water bath. If during the evaporation a coagulum formed it was filtered off. The evaporation was continued until a volume of about 15 cc. was obtained. The extract was then ready to be introduced into the nitrate apparatus. The size of sample taken was ordinarily 30 to 50 gm. In case of foods known to contain only traces of nitrate, this sample at times weighed as high as 100 gm. In analyzing milk, 200 cc. were taken and the protein was precipitated with trichloroacetic acid, filtered off, and washed thoroughly with water. The filtrate was then evaporated on the water bath to a small volume and was then ready for analysis. All analyses were run in duplicate or triplicate.

5. In the analysis of urine, 200 cc. portions were taken in most cases, except with the urines in the experiments on rats. The samples were then made alkaline, unless already in that condition, and evaporated to about 25 cc. on the water bath. They were then treated with 75 cc. of 75 per cent alcohol, filtered, the precipitate was carefully washed with 75 per cent alcohol, and the filtrate concentrated on the water bath to about 10 to 15 cc. They were then ready for analysis and gave no trouble by foaming. These analyses were also run in duplicate or triplicate whenever possible. With the rat urines it was necessary to take the entire collection for a single analysis.

The Accuracy of the Nitrate Method.

The accuracy of all methods based on the reduction of nitric acid to nitric oxide by ferrous chloride, in the presence of organic matter, has been the subject of considerable investigation. These methods have proven to be especially valuable in the analysis of soils, urine, animal fertilizer, and a wide variety of plant and animal material. The results of most analysts have indicated the trustworthiness of the methods in the presence of urea, ammonium salts, proteins and protein derivatives, organic acids, etc., and, in general, good results have been reported on the analysis of urine and other materials containing organic substances.

However, some results have been reported which indicate a disturbing effect of urea and ammonium salts in particular. Pfeiffer and Thurmann, and Pfeiffer[38] have concluded from a large number of analyses that the presence of urea and ammonium salts reduces the theoretical yield of nitric oxide. On the other hand, Seydel and Wichers[39] conclude that urea increases the theoretical yield of nitric oxide. Opposed to the conclusions of all these analysts stands the excellent work of Warington,[40] Liechti and Ritter,[38] and the analyses reported in Tiemann-Gärtner's Handbuch[34] (page 221), according to which urea and ammonium salts exert no demonstrable effect on the reduction of nitrates to nitric oxide.

Working with pure nitrate solutions (samples containing 50 to 150 mg. of KNO_3), we have found that from 95 to 100 per cent of the theoretical quantities of nitric oxide may be recovered under the most favorable conditions, using the method outlined above.[41] As a general rule, in agreement with other investigators,

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[38] Pfeiffer, T., and Thurmann, H., *Landw. Vers.-Stat.*, 1895, xlvi, 1. Pfeiffer, T., *Z. anal. Chem.*, 1903, xlii, 612. For a good review of the literature on this point see Liechti, P., and Ritter, E., *Z. anal. Chem.*, 1903, xlii, 205.

[39] Seydel, S., and Wichers, L., *Z. angew. Chem.*, 1911, xxiv, 2046.

[40] Warington, R., *J. Chem. Soc.*, 1880, xxxvii, 468; 1882, xli, 345; also cited by Hintz, E., *Z. anal. Chem.*, 1884, xxiii, 545.

[41] In this work on pure nitrate solutions, the gas collected was not treated with the permanganate solution but was measured directly. It is therefore comparable to the results of other investigators.

we have obtained more satisfactory results with the larger quantities of nitrate. The presence of a large excess of urea (twenty to thirty times the weight of nitrate taken) appeared to exert no definite effect on the volume of gas obtained, though in general it was more difficult to obtain good duplicates in the presence of this substance. We did not investigate the influence of ammonium salts, since our method of preparing the food and urine samples for analysis involved the decomposition of all ammonium salts present.

In our first analyses of human urine, four samples were taken from each collection, three were analyzed for nitrates directly, and the fourth after the addition of a definite weight of pure potassium nitrate. From the results obtained with nineteen samples of urine to which from 22 to 194 mg. of potassium nitrate were added, it was found that an average of 92.4 per cent of the added nitrate could be accounted for by the quantity of NO gas obtained. With 10 samples of urine, each consisting of 200 cc., to each of which 10.111 mg. of KNO_3 were added, an average of 93.2 per cent of the added nitrate was recovered.

The lower percentage of added nitrate recovered from urine as compared with distilled water solutions is in agreement with the results of Liechti and Ritter. These analysts were able to recover an average of 98.2 per cent of the nitrate in water solution (fourteen analyses), and an average of 94.8 per cent in urine solution (eleven analyses). In the method used by Liechti and Ritter, the total gas collected not absorbed by alkali was measured and calculated as NO. This fact may account for the slightly larger average recovery of nitrate added to urine of known nitrate content.[42] It is evidently a fact that the method is not as satisfactory in the analysis of urine as in the analysis of pure nitrate solutions, but to what constituent of urine the disturbing effect is due cannot at present be said. It was also our experi-

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[42] The average residual gas unabsorbed by the alkaline permanganate solution for the urine analyses was 0.25 cc. (160 analyses). The amount of residual gas was not obviously correlated with the amount of total gas obtained. In the food analyses larger residues were obtained, i.e., about 0.50 cc., while in the case of the analysis of milk, high values were always obtained, averaging about 0.80 cc.

ence that it is more difficult to obtain satisfactory duplicates with urines than with pure nitrate solutions. Our analyses of human urine and pig's urine were all run in triplicate, and if good agreement was not obtained, a second analysis in duplicate or triplicate was run whenever possible. In this connection, we determined by repeated tests that thymol and refrigeration will preserve urine for at least 5 days at constant nitrate content.

TABLE I.

Analytical Results on the Recovery of Potassium Nitrate Added to Wheat Bread and Boiled Potato.

Food used.	KNO_3 added.	KNO_3 recovered.	Recovery.
	mg.	*mg.*	*per cent*
25 gm. bread................	22.0	17.0	77.3
25 " "	24.1	19.2	79.7
25 " "	56.2	46.5	82.7
25 " "	53.4˙	37.5	70.2
25 " "	81.9	62.1	75.8
25 " "	78.8	61.6	78.2
25 " "	132.2	99.7	75.4
25 " "	142.6	90.8	63.7
			Average, 75.4
35 gm. potato...............	24.2	20.2	83.5
35 " "	23.7	23.5	99.2
35 " "	142.5	110.0	77.2
35 " "	131.0	111.7	85.3
			Average, 86.3

In testing the method for the analysis of nitrates in foods the results obtained were not as satisfactory as those obtained from the tests with urine. In Table I are given the data resulting from adding definite amounts of potassium nitrate to bread and boiled potatoes, extracting in the usual way, and determining the nitrate in the concentrated and filtered extract. The extraction of the foods was continued until the diphenylamine test applied to the extracts was negative.

The figures given in the third column of the table were obtained after due allowance was made for the nitrate content of the foods

used.[43]   Evidently better results were obtained with the potatoes than with the bread.[44]

These tests indicate that a smaller percentage of the nitrate added to foods is recovered by the nitrate method used than in the case of the addition of nitrate to urine.   This is very probably due to the reduction of some of the nitrate by the soluble carbohydrates of foods.   Thus, Warington[40] found that the addition of sugar to a nitrate solution lowered the percentage recovery of the nitrate, a result also noted by Schloesing.[45]

However, this reducing action of carbohydrates on nitrates may be more than compensated by the production of other gases in the decomposition flask, among which Warington has found carbon monoxide as an important constituent.   Warington found that, in the presence of carbohydrates, much larger volumes of residual gases, unabsorbable by soda solution or a solution of a ferrous salt, resulted than in their absence.   Our experience has abundantly confirmed the production of gases from the action of carbohydrates on ferrous chloride and hydrochloric acid.   Thus three 10 gm. samples of sucrose were analyzed by the method outlined in this paper, with the production of 0.87, 1.02, and 0.81 cc. of gas.   Upon shaking this gas in the Hempel pipette with the alkaline permanganate solution, these volumes were gradually reduced to 0.80, 0.91, and 0.69 cc., respectively.

The reduction in volume noted was probably due to the oxidation of the CO gas present and the retention of the CO_2 in the pipette.   To test the relative absorption of NO and CO by the alkaline permanganate solution the following experiment was undertaken.   Nitric oxide gas was produced by the action of ferrous chloride and hydrochloric acid on pure KNO_3.   This gas

[43] The bread analyzed 0.196 mg. KNO_3 per 25 gm., or 0.0008 per cent. The potato gave 2.45 mg. KNO_3 per 35 gm., or 0.007 per cent.

[44] An interesting though unexplained observation was made in evaporating the water extracts of the bread samples on the water bath after they had been made alkaline.   At first they were all of a light yellow color, but as digestion proceeded, they all darkened, though unequally.   On several hours' heating, those samples containing no added nitrate were dark brown in color, those containing the most added nitrate were lightest in color, while the gradations in color between these extremes were roughly proportional to the quantity of added nitrate.

[45] Schloesing, T., *Ann. chim. phys.*, 1854, series 3, xl, 479.

was shaken for successive 30 second intervals in the Hempel pipette with alkaline permanganate solution and the absorption followed by measuring the residual gas between these intervals. The results of three experiments are given below:

	I.	II.	III.
	cc.	cc.	cc.
Original volume of NO	28.50	39.70	31.40
Volume after 30 seconds' treatment	0.25	1.00	0.65
" " 60 " "	0.20	0*95	0.55
" " 90 " "	0.15	0.90	0.50
" " 120 " "	0.15	0.90	0.50

Evidently the NO is rapidly absorbed, complete absorption resulting after 90 seconds' shaking with the absorbent. The same sort of a test was run on CO, obtained by the action of H_2SO_4 on oxalic acid and collection of the gas over alkali. The intervals of shaking were in this case 4 minutes:

	I.	II.
	cc.	cc.
Original volume of CO	38.05	8.87
Volume after 4 min. treatment	31.20	7.31
" " 8 " "	22.70	5.95
" " 12 " "	15.50	4.20
" " 16 " "	7.80	3.57
" " 20 " "	4.80	2.50
" " 24 " "	3.10	2.08
" " 28 " "	2.50	1.76
" " 32 " "	2.00	1.57

This gradual absorption continued until at the end of 52 minutes the first volume was reduced to 1.30 cc., and at the end of 68 minutes the second volume was reduced to 1.05 cc.

It would seem, therefore, that the Schulze method for the determination of nitrates would tend to give too high results in the analysis of foods, especially foods containing much starch or sugars and small amounts of nitrates, and in particular would indicate a much wider distribution of nitrates in food materials than actually exists. Thus, in the experiments on pigs, reported

below, the three foods used when analyzed by the Schulze method gave results indicating a fairly considerable content of nitrate, though a color test for nitrates in the original foods and a test for NO in the gas produced by the action of ferrous chloride and hydrochloric acid, gave entirely negative results.

At times the nitrate determinations on the foods used were extremely erratic. Thus, one sample of cheese on toast, a food used in the first experiment, gave the extreme value of 0.0931 per cent of nitrate as KNO_3, while a repetition of the determination gave 0.00019 per cent, a value consistent with the results obtained on the bread and cheese analyzed separately. Such impossible values as were very occasionally obtained were possibly due to an abnormal production of carbon monoxide. In every case before discarding these data other analyses were made to make sure that they were in fact erratic.

Experiments on Men.

The first subject (H. H. M.) whose nitrate balance was determined was a man about 30 years of age and 68.5 kg. body weight. During the course of the experiment this subject was performing office and laboratory work and was taking his meals at home. From July 20 to 24 (1915) inclusive, preliminary observations were made on the daily excretion of nitrates in the urine. While the subject was on an unrestricted diet, the total quantities of nitrates excreted per day in the urine, expressed as mg. of KNO_3, for these 5 days, and the total volumes of urine per day, were:

	Potassium nitrate.	Urine.
	mg.	*cc.*
July 20	141	1,815
" 21	279	1,475
" 22	98	1,870
" 23	85	1,360
" 24	632	1,390

The irregularity of the excretion depended upon the variable quantity of fresh vegetables eaten. Thus, the high value for July 24 was due to a liberal consumption of beets, which are known to possess a very high content of nitrates.

From July 30 to Aug. 12, inclusive, the subject confined himself to a diet free from fresh vegetables, and consisting largely of potatoes, milk, bread, wheat cakes, crackers, meat, and eggs. During the last 7 or 8 days of this period, potatoes were omitted from the diet, since they were found to contain appreciable quantities of nitrates. The daily excretions of nitrates in the urine were:

	Potassium nitrate.	Urine.
	mg.	cc.
July 30	146	1,400
" 31	100	980
Aug. 1	133	1,130
" 2	101	1,190
" 3	119	1,060
" 4	91	1,240
" 5	81	1,215
" 6	68	1,065
" 7	71	1,240
" 8	80	1,010
" 9	67	1,080
" 10	62	1,485
" 11	74	1,400
" 12	49	1,435

It is thus seen that the restricted diet resulted in a lowered excretion of nitrates, which was fairly constant for the last week or so of the period.

During the 4 days from Aug. 13 to 16, inclusive, the subject was on a weighed diet. A sample of all articles of food eaten was reserved for analysis. The water drunk was mainly distilled water, though small amounts of city water were used also. The latter contained small quantities of nitrates, equivalent on an average to about 1 mg. of potassium nitrate per liter.

The food consumed per day during the 4 day period was as follows:

Aug. 13   Wheat cakes, 251 gm.   Beefsteak, 140 gm.
          Milk, 1,500 cc.   Cheese on toast, 157 gm.
          Eggs, 1   Bread, 168 gm.

" 14   Wheat cakes, 309 gm.   Lamb chops, 117 gm.
          Milk, 1,250 cc.   Bread, 179 gm.
          Eggs, 2   Cheese, 44 gm.

Aug. 15 Wheat cakes, 287 gm.   Cheese on toast, 226 gm.
         Milk, 850 cc.   Bread, 263 gm.
         Eggs, 2

"   16   Eggs, 3   Bread, 316 gm.
         Milk, 1,000 cc.

The sugar and butter used were not weighed, nor were small amounts of gravy eaten on Aug. 13 and 14. The bread was eaten fresh or toasted. The meat was not analyzed, but was assumed to be nitrate-free in accordance with the analyses of other investigators.

The nitrate balance of Subject H. H. M. during this period is given in Table II.

TABLE II.

Nitrate Balance of Subject H. H. M.

Date.	Volume of urine.	Nitrate values expressed as mg. of KNO₃.		
		Nitrate intake.	Nitrate in urine.	Nitrate balance.
	cc.			
Aug. 13	1,315	13	61	−48
" 14	1,180	13	51	−38
" 15	917	12	64	−52
" 16	1,175	11	60	−49
Average		12	59	−47

From these data it appears that the subject was excreting in the urine, in a fairly constant manner, about five times as much nitrate as he was ingesting.

The second subject (H. A. S.) was 23 years of age and weighed about 64 kg. For 8 to 10 hours a day this subject was working in the laboratory. On July 21, a day's collection of urine was found to contain 345 mg. of nitrate calculated as KNO₃. The diet at this time was unrestricted and included the usual amount of fresh vegetables. From Aug. 17 to 30, inclusive, the subject was on a weighed diet, consisting as far as possible of foods o of low nitrate content, such as shredded wheat biscuit, corn-starch pudding, milk, eggs, and meat. The food intake during this 2 week period is given in Table III.

TABLE III.

Food Intake in the First Experiment with Subject H.A.S.   Weights of Solid Foods Expressed in Gm.   Milk Intake Expressed in Cc.

Food.	Aug. 18	19	20	21†	22	23	24	25	26	27	28‡	29	30§
Shredded wheat biscuit..........	112	84	84	84	70	56	112	140	112	84	70	28	28
Whole wheat bread.	185	220	165	177	196	173	268	69	181	295			
White bread........								221	57		235	260	320
Whole milk.........	300	225	225	225	100	150	300	375	300	1,172	1,325	75	75
Buttermilk.........	2,000	1,673	1,973	2,073	1,700	1,847	900	1,700	1,373				
Eggs*..............	2	2	2	1	1	1	3	2	2	4	5	3	2
Corn-starch pudding..............	76	119	111	77	352	320		150					
Beef loaf..........	164			129	126	76							
Other meat........		110	90				87	90	73	64		150	
Cake..............											20	80	

* The consumption of eggs is indicated by the number eaten.
† On this day a dish of ice-cream was eaten. It was not weighed nor was a sample analyzed.
‡ On Aug. 28 there was an extra food consumption of one bar of Hershey's chocolate and one dish of Krumbles.
§ On Aug. 30, besides the foods given in the table, there were consumed 150 gm. of potatoes, 150 gm. of macaroni and cheese, and an unweighed amount of apple butter.

All foods eaten during this period were sampled and analyzed with the exception of the meat other than beef loaf, the cake, and the extra foods mentioned in the foot-notes to the table. Therefore, the nitrate intake for the last 4 days of the period was only approximately determined.

On Aug. 25, 986 mg. of KNO₃ were taken with the food, being distributed among the three meals. In Table IV are given the data of the intake of nitrate (figured as KNO₃), the excretion in the urine, the daily volume of urine, and the apparent nitrate balance.

During this 2 week period the subject was evidently excreting much more nitrate than he was ingesting, generally four to five times as much, and on some days even a greater ratio than this existed. After the ingestion of the potassium nitrate added to the food on Aug. 25, apparently only 60 to 65 per cent was recovered in the urine.

A second experiment on H. A. S. was run from Sept. 24 to Oct. 2, inclusive. During this 9 day period the food intake was under much better control than in the first experiment, being restricted

TABLE IV.

Nitrate Balance in the First Experiment on Subject H. A. S.

Date.	Volume of urine.	Nitrate values expressed as mg. of KNO₃.		
		Nitrate intake.	Nitrate in urine.	Nitrate balance.
	cc.			
Aug. 17	845		57	
" 18	1,545	22	66	−44
" 19	1,125	20	73	−53
" 20	1,475	21	88	−77
" 21	1,615	21	108	−87
" 22	790	20	58	−38
" 23	900	20	79	−59
" 24	820	16	83	−67
" 25	1,515	1,011	588	+423
" 26	1,465	19	169	−150
" 27	1,280	15	108	−93
" 28	1,095	17	120	−103
" 29	1,360	10	154	−144
" 30	2,310	12	90	−78

to eggs, shredded wheat biscuit, corn-starch pudding,[46] milk, and sugar. All foods were carefully weighed out and analyzed. The food intake is given in Table V.

TABLE V.

Food Intake in the Second Experiment on Subject H. A. S.

Food	Sept. 24	25	26	27	28	29	30	Oct. 1	2
Shredded wheat biscuit, gm	280	218	189	273	276	224	258	316	1.
Eggs	2	2	2	0	0	0	0	0	4
Corn-starch pudding, gm	250	531	210	510	627	955	975	977	
Milk, cc	1,184	1,178	946	1,419	1,419	1,419	1,419	1,892	1,
Sugar, gm	75	80	55	75	85	89	90	90	

On Sept. 26 approximately 1 liter of city water was consumed and on Sept. 30, 200 cc. of city water were taken. Otherwise

[46] The corn-starch pudding was made according to the following recipe 1 quart of milk, 65 gm. of corn-starch, 3 eggs, seasoned with chemically pure NaCl.

only distilled water was used.   On Sept. 22 and 23 the food intake was restricted so that all fresh vegetables were excluded from the diet.   Also the urine was collected on these days.

In Table VI are given the data on the nitrate balance during this period, and also the daily volumes of urine.

TABLE VI.

Nitrate Balance in the Second Experiment on Subject H. A. S.

Date.	Volume of urine.	Nitrate values expressed as mg. of KNO₃.		
		Nitrate intake.	Nitrate in urine.	Nitrate balance.
	cc.			
Sept. 22	745		145	
" 23	978		146	
" 24	670	9.4	65	−56
" 25	786	10.6	89	−78
" 26	674	7.6	63	−55
" 27	830	11.5	62	−51
" 28	825	12.1	60	−48
" 29	1,045	13.7	62	−48
" 30	1,300	13.9	51	−37
Oct. 1	1,326	16.8	73	−56
" 2	845	12.1	92	−80

TABLE VII.

Nitrate Content of Foods Used.

Nitrate Values Expressed as Percentage of KNO₃.

Food.	KNO₃	Experiment.	Food.	KNO₃	Experiment.
	per cent			*per cent*	
Wheat cakes	0.00090	H. H. M.	Bread (whole wheat)	0.0023	H. A. S.
Eggs	0.00061*	H. H. M. and H. A. S.	Bread (white)	0.0033	H. A. S
Bread (white)	0.0017	H. H. M.	Milk	5.6 mg. KNO₃ per 1	H. A. S.
Milk	4.9 mg. KNO₃ per 1	H. H. M.	Corn-starch pudding	0.00053	H. A. S
Cheese on toast	0.00019	H. H. M.	Buttermilk	7.2 mg. KNO₃ per 1	H. A. S.
Cheese	0.0011	H. H. M.	Beef loaf†	0.00022	H. A. S.
Potato	0.00023	H. A. S.			
Shredded wheat biscuit	0.00033	H. A. S.			

* This is equivalent to 0.28 mg. KNO₃ per egg.   It was considered sufficiently accurate in this work to use this figure applied to the number of eggs consumed.

† Made from beef, eggs, and crackers.

In this experiment, as in the preceding two, the nitrate excretion in the urine is several times larger than the intake.

The nitrate contents of the foods used in the above three experiments are given in Table VII.

Experiments on Rats.

Experiments were run on four adult albino rats to determine the nitrate balance, from Aug. 24 to Sept. 10, inclusive. For several weeks previous to this period, and during the experimental period, the rats were kept upon a ration consisting of the following food materials:

	per cent
Whole milk powder	54
Corn-starch	18
Lard	28

Upon the analysis of 10 gm. portions of this ration it was found to contain nitrate equivalent to 0.000166 per cent of KNO_3.

The rats were each kept in a round galvanized iron cage, 8 inches in diameter and 6 inches high. These cages were firmly supported in short-stem glass funnels with a maximum diameter of 10.5 inches, which in turn were supported in a wooden frame. Beneath each funnel was placed a wide-mouthed bottle with a capacity of about 10 ounces, for the collection of the urine. Both funnels and bottles were rinsed with an alcoholic solution of thymol, and a few crystals of thymol were placed in each bottle, immediately before each day's collection. At the end of 24 hours, the rats were removed from the cages, and cages and funnels were carefully washed down with boiling water, the washings being collected in the proper bottle. The feces were removed from the cages before this washing, and any contamination of the urine with feces was avoided as much as possible by placing in each funnel a small wad of glass wool, which was rinsed with boiling water in the same manner as the funnels and cages, after the removal of adhering feces.

The data of this experiment are contained in Table VIII.

From Aug. 25 to 28, inclusive, and Aug. 30 to Sept. 2, inclusive, the urines were analyzed as 4 day composite samples. On all other days the daily collections were analyzed separately. Each

collection of urine, whether for 1 day or for 4 days, was used for one analysis.

The data as a whole indicate strongly that the rats were consistently excreting in the urine more nitrate than they were ingesting. We believe that the two 4 day periods above mentioned offer the most reliable data on this point, since the urine collec-

TABLE VIII.

Experimental Data on the Nitrate Balance of Albino Rats.
Nitrate Values Expressed as Mg. of KNO₃.

Date.	Rat 1 ♂—133.9 gm.			Rat 2 ♀—127.6 gm.			Rat 3 ♀—125.1 gm.			Rat 4 ♂—176.7 gm.		
	Food eaten.	Nitrate intake.	Nitrate in urine.	Food eaten.	Nitrate intake.	Nitrate in urine.	Food eaten.	Nitrate intake.	Nitrate in urine.	Food eaten.	Nitrate intake.	Nitrate in urine.
1915	gm.			gm.			gm.			gm.		
Aug. 24	5.6	0.093	1.31	2.9	0.048	1.27	4.9	0.081	0.52	6.7	0.111	0.11
" 25	4.7			4.4			5.5			6.3		
" 26	5.2	0.388	9.77	5.5	0.370	3.74	5.4	0.392	3.00	7.3	0.468	2.68
" 27	5.5			6.3			5.8			9.1		
" 28	8.0			6.1			6.9			5.5		
" 29	5.3	0.088	—*	5.0	0.083	—*	6.4	0.106	—*	8.3	0.138	—*
" 30	5.9			6.8			6.3			7.4		
" 31	4.9	0.364	3.45	5.5	0.364	1.50	6.7	0.403	0.80	5.9	0.476	0.88
Sept. 1	5.2			4.8			5.8			7.0		
" 2	5.9			4.8			5.5			8.4		
" 3	2.9	60.55†	32.36	3.1	80.95†	Lost.	3.2	153.45†	45.50	1.8	61.53†	Lost.
" 4	4.4	0.073	4.01	5.5	0.091	7.86	5.0	0.083	22.35	5.2	0.086	10.02
" 5	5.3	0.088	0.90	4.3	0.071	0.04	6.5	0.108	0.43	6.3	0.105	0.23
" 6	6.5	0.108	—‡	5.7	0.095	—‡	6.1	0.101	—‡	5.9	0.098	—‡
" 7	4.0	43.07†	26.12	3.8	80.36†	53.28	3.9	40.81†	25.43	2.6	48.04†	23.79
" 8	5.0	0.083	3.28	6.0	0.100	4.97	6.1	0.101	4.28	4.5	0.075	1.33
" 9	5.9	0.098	1.71	5.8	0.096	0.20	6.8	0.113	0.46	5.4	0.090	0.27
" 10	4.0	0.066	1.53	5.6	0.093	0.13	5.8	0.096	0.19	3.1	0.051	0.22

* All urines of Aug. 29 were submitted to a qualitative test for nitrate; the diphenylamine test was used. The results were all positive.

† KNO₃ added to food.

‡ All urines of Sept. 6 were also examined qualitatively for nitrates. No clearly positive results were obtained.

tions contained more nitrate, and the error in its determination was, therefore, less than was the case with the daily periods.

On Sept. 3 and again on Sept. 7, varying amounts of KNO₃ were added to the rations of the rats, and in all cases marked increases in the excretion of nitrate in the urine resulted, which persisted over 2 days. The percentage recovery of nitrate in the urine varied from about 45 to 65.

Experiments on Pigs.

Four pure-bred Chester-White pigs were the subjects of the final experiment on the nitrates in the urine.[47] For over 6 months these pigs had been on a feeding experiment and had been in continuous confinement in metabolism stalls. Two were being fed corn-meal and pasteurized skim milk, and the other two rolled wheat and pasteurized skim milk. All water drunk throughout the entire experiment was distilled. Three of the pigs were not in good condition and had been consistently losing in weight for 5 weeks previous to the beginning of the nitrate experiment. About 2 weeks after the close of this test, they were killed and a postmortem examination was made of the carcasses. Several of the internal organs were in an atrophied condition. The fourth pig was in good condition, considering his long confinement, and had gained in weight continuously from the beginning of the experiment.

The nitrate balance of the pigs was determined during the 4 days from Dec. 29, 1915, to Jan. 1, 1916, inclusive. On Jan. 1, the three abnormal pigs weighed 195.0, 173.5, and 190.5 pounds; the normal pig weighed 271.0 pounds.

The three feeds used in this experiment were thoroughly sampled and analyzed for nitrate. The results of the analysis need some explanation. The corn and wheat were analyzed in four 50 gm. samples each, the milk in four 200 gm. samples. The gas obtained from one sample out of each set of four was mixed with a small quantity of air and shaken with a potassium iodide starch solution. The presence of nitric oxide in the gas obtained would lead to the formation of nitrous acid under the conditions of the test, which would liberate the iodine from the KI with the formation of the intense blue color of starch iodide. According to Richardson,[48] this is a very sensitive test. The tests on the corn and wheat were entirely negative, no coloration at all being produced. The test on the milk gave a very faint coloration. A second test which was run on the skim milk was

entirely negative. Water extracts of portions of the corn and wheat also gave no coloration with the diphenylamine test for nitrates; the same result was obtained when this test was run on the filtrate obtained from a sample of the milk after the removal of the proteins by trichloroacetic acid.

The volumes of gas obtained in the course of the regular nitrate method, when shaken with the alkaline permanganate solution, showed decreases, which when figured as nitric oxide gave the following percentages of KNO_3: corn, 0.00496; wheat, 0.0157; and milk, 0.00378. These percentages are comparatively large, but, in view of the negative character of the qualitative tests above mentioned and of the marked tendency of starchy foods to produce other gases than NO absorbable by an alkaline permanganate solution on treatment with ferrous chloride and hydrochloric acid, we believe they are very deceptive. In Table IX are included the food intake, the nitrate intake as calculated from the percentages just given, the volume of urine, and the nitrate in the urine for the four pigs during the 4 days of observation. The food intake of the immediately preceding 5 days is also given.

The following urine collections were submitted to the KI starch test as described above:

Dec. 29 Urine collections of Pigs 315 and 815.
"   30   "   "   "   215 and 815.
Jan. 1 "   "   "   215, 315, and 415.

All of these tests were distinctly positive. No negative tests were obtained on the urines of this experiment.

The data of Table IX afford good evidence that the values given in the table for the nitrate intake, calculated from the volume of gas absorbed by the alkaline permanganate solution, are much too high. This evidence consists of the fact that the much larger feed intake of Pig 815 was not accompanied by anything approaching a correspondingly large nitrate excretion. Everything considered, it seems safe to assume that the feeds of this experiment were nitrate-free, with the possible exception of the milk, but that in the urine small quantities of nitrate were excreted.

The relatively small volumes of urine secreted by the three abnormal pigs, especially by Pig 315, are remarkable. This, of course, necessitated taking smaller samples of urine for analysis. The urine of Pig 815 was analyzed in 400 cc. samples.

TABLE IX.

Nitrate Balance of Pigs. *

Nitrate Values Expressed as Mg. of KNO₃.

Date.	Pig 215. Weight on Jan. 1, 195.0 lbs.					Pig 315. Weight on Jan. 1, 173.5 lbs.				
	Corn intake.	Milk intake.	Nitrate intake.	Volume of urine.	Nitrate in urine.	Wheat intake.	Milk intake.	Nitrate intake.	Volume of urine.	Nitrate in urine.
1915	lbs.	lbs.	mg.	cc.	mg.	lbs.	lbs.	mg.	cc.	mg.
Dec. 24....	1.0	2.0				1.0	2.0			
" 25....	1.5	3.0				0.5	1.0			
" 26....	1.0	2.0				0.5	1.0			
" 27....	1.0	2.0				1.0	2.0			
" 28....	1.2	2.4				1.2	2.4			
" 29 ...	1.0	2.0	56.9	264	7.1	1.0	2.0	105.7	602	21.6
" 30...	1.0	2.0	56.9	538	32.0	1.0	2.0	105.7	147	7.0
" 31....	0.5	1.0	28.4	408	8.8	0.5	1.0	52.8	0	0.0
Jan. 1....	1.0	2.0	56.9	335	11.6	1.0	2.0	105.7	375	15.0

Date.	Pig 415. Weight on Jan. 1, 190.5 lbs.					Pig 815. Weight on Jan. 1, 271.0 lbs.				
	Wheat intake.	Milk intake.	Nitrate intake.	Volume of urine.	Nitrate in urine.	Corn intake.	Milk intake.	Nitrate intake.	Volume of urine.	Nitrate in urine.
1915	lbs.	lbs.	mg.	cc.	mg.	lbs.	lbs.	mg.	cc.	mg.
Dec. 24....	1.0	2.0				4.5	9.0			
" 25....	0.5	1.0				4.5	9.0			
" 26....	0.5	1.0				4.5	9.0			
" 27....	0.5	1.0				4.5	9.0			
" 28....	0.5	1.0				4.5	9.0			
" 29....	1.0	2.0	105.7	745	21.3	4.5	9.0	742.9	3,005	51.1
" 30....	0.0	0.0	0.0	418	19.1	4.5	9.0	742.9	2,318	33.4
" 31....	0.5	1.0	52.8	524	16.9	4.5	9.0	742.9	3,315	23.0
Jan. 1....	1.0	2.0	105.7	622	17.9	4.5	9.0	742.9	2,490	14.0

* The nitrate intake values were calculated from the percentages of KNO₃ in the feeds on the assumption that all of the gas collected and absorbable by the alkaline permanganate solution was NO. According to qualitative color tests, however, the feeds were nitrate-free, with the possible exception of the milk.

CONCLUSIONS.

The experiments above described indicate, in most cases beyond reasonable doubt, that the animal body may excrete in the urine more nitrate than is ingested in the food. The marked differences between the determined nitrate intake and nitrate excretion cannot be accounted for by differences in the accuracy of the method as applied to foods and to urine. The evidence indicates that the urinary nitrates may be determined by our method in a fairly satisfactory manner, since pure KNO_3 added to urines of determined nitrate content could be recovered on an average to the extent of 92 to 94 per cent. The nitrate content of foods cannot be determined in such a satisfactory manner. Nitrates added to foods of determined nitrate content can only be recovered to the extent of 75 to 85 per cent, probably due to the reducing action of sugars. However, this deficit as compared with the equivalent values obtained on urines is probably more than compensated for by the production of gases other than nitric oxide which are retained by the absorbent used, upon treatment with ferrous chloride and hydrochloric acid. This fact is clearly indicated by the analyses in the experiments on pigs.

It seems reasonable to believe, therefore, that the values given for the intake of nitrate in our experiments are high, rather than low, and since there is no reason to doubt the accuracy of the values for the nitrate content of the urine except that they are slightly low, the evidence for the conclusion that the nitrate of the urine is not derived entirely from the food in the case of the subjects experimented upon seems convincing. This conclusion is also supported by the fact, which our own data confirm, that ingested nitrates are destroyed in the body to the extent of 40 to 60 per cent.

There are two possible explanations of the source of the excess of nitrate appearing in the urine over the nitrate ingested with the food. It is conceivable that the nitric and nitrous acids in the air may gain access to the blood in the lungs and ultimately find their way to the urine. Some investigations by Lassar,[49] confirmed by Spiegel,[50] would indicate, however, that nitric acid

[49] Lassar, O., *Z. physiol. Chem.*, 1877-78, i, 165.
[50] Spiegel, A., Ueber die Ausscheidung der Salpetersäure, Inaug. Dissertation, Würzburg, 1894.

fumes are irrespirable. Thus, Lassar showed that if dogs and
rabbits on a nitrate-free diet were exposed to concentrated nitric
acid fumes, the urine was nitrate-free. Furthermore, assuming
a perfect permeability of the lungs to nitric acid, the quantity
in which it occurs in the air seems entirely too small to account
for the discrepancies observed between the nitrate ingested with
the food in our experiments and the nitrate eliminated in the
urine. Thus, taking Mabery's[51] figures obtained from analyses
of samples of air taken at Cleveland, it may be shown that the
total quantity of nitric acid that would be introduced into the
lungs per day, on the assumption that the tidal air is 500 cc. and
the respiration rate 20 per minute, would average about 5 mg.,
the maximum being 15.3 mg. The quantity of nitrous acid
inhaled is entirely negligible.

The other possible explanation is that the body tissues are
able to produce nitrates from unoxidized nitrogenous radicles.
It would be mere speculation to attempt a theory of the manner
in which this oxidation is accomplished, the intermediate products
formed, the particular organ responsible for it if its production
is at all localized, or of the biological significance of the process.
Such speculations would be profitless in the present state of our
knowledge on this question. We hope to investigate the matter
further along these lines. We believe, however, that the produc-
tion of nitrates by animal tissues is a fact and is unique in being
the only well established instance of the oxidation of nitrogen,
aside from the conversion of nitrites to nitrates, which is appar-
ently a reversible reaction.

[51] Mabery, C. F., *J. Am. Chem. Soc.*, 1895, xvii, 105.

THE DIETARY FACTORS OPERATING IN THE PRODUC-
TION OF POLYNEURITIS.*

By E. V. McCOLLUM and CORNELIA KENNEDY.

*(From the Laboratory of Agricultural Chemistry of the University
of Wisconsin, Madison.)*

Eykman,[1] Suzuki,[2] Funk,[3] and others have made it clear that
continued feeding of a diet of polished rice induces polyneuritis
in birds. Funk[4] has likewise shown that identical results come
from feeding birds diets of highly purified foodstuffs. This
pathological condition, he has shown, is relieved by the introduc-
tion into the diet of water or alcoholic extracts of several natural
foodstuffs.

The studies of a number of investigators have now fully estab-
lished the fact that animals cannot grow when limited to rations
of carefully purified proteins, carbohydrates, fats, and salts.[5]
McCollum and Davis[6] have shown that certain fats, as butter
fat, egg yolk fat, kidney fat, and others contain something which
greatly stimulates growth when added to a diet of casein, dextrin,
lactose, and a salt mixture of appropriate composition. This
substance is present in certain products of plant origin as well.
Later they discovered that if the lactose was replaced by dextrin

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* Published by permission of the Director of the Agricultural Experi-
ment Station.

[1] Eykman, C., *Arch. path. Anat.*, 1897, cxlviii. 523; cxlix, 187; *Arch.
Hyg.*, 1906, lviii, 150.

[2] Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89.

[3] Funk, C., *Ergebn. Physiol.*, 1913, xiii, 125, gives an extensive résumé of
the literature relating to the so called deficiency diseases, together with a
complete bibliography of the older literature.

[4] Funk, *Z. physiol. Chem.*, 1914, lxxxix, 374.

[5] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425. Hopkins, F. G., and
Neville, A., *Biochem. J.*, 1913, vii, 97. Funk, C., and Macallum, A. B.,
Z. physiol. Chem., 1914, xcii, 13. Stepp, W., *Z. Biol.*, 1913, lxii, 405.

[6] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 179.

in this ration the addition of butter fat did not induce growth. When this diet, containing butter fat, was supplemented with small additions of the water extract of boiled egg yolk, or water or alcoholic extracts of wheat germ, growth proceeded at the normal rate. They formulated the working hypothesis that in addition to the recognized essential constituents of a successful diet, *viz.*, protein, carbohydrate, fats, and inorganic salts, the growing animal requires two substances, or groups of substances; one, which is soluble in fats, is contained in fats from certain sources; the other, which is soluble in water and alcohol, is found widely distributed both in the animal and vegetable worlds.

Funk and Macallum[7] have pointed out that butter fat does not relieve polyneuritis in pigeons and also that with rations consisting, aside from the butter fat content, of carefully purified foodstuffs, does not induce growth in young rats and they clearly express their belief that "vitamines" are absent from butter fat.

By "vitamine" Funk and his coworkers indicate a class of substances the chemical nature of which is at present unknown. They are described as being precipitated by phosphotungstic acid and by mercuric chloride, but aside from this property there is no evidence that these substances of unknown constitution which are active in promoting growth, or in relieving the symptoms of polyneuritis contain an amino group. Furthermore, the prefix *vita* connotes an importance in biological processes paramount to that of certain other absolutely indispensable organic complexes, among which are a number of amino-acids. We feel that this term is not in harmony with a conservative tendency in the nomenclature of biological chemistry which should avoid the employment of a term which carries the idea that one indispensable complex is of greater importance biologically than another one equally indispensable. Furthermore, the evidence of the presence of an amino group in the substances under consideration is too slight to warrant the use of the ending *amine*, which carries with it a definite meaning in organic chemical nomenclature.

Hopkins[8] introduced the term "accessory" for the substances of unknown chemical character which are found in milk and which

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[7] Funk and Macallum *J. Biol. Chem.*, 1915, xxiii, 413.

[8] Hopkins, *J. Physiol.*, 1912, xliv, 425. Hopkins and Neville, *Biochem. J.*, 1913, vii, 97.

render the addition of small amounts of milk to rations consisting otherwise of purified food substances, so effective in promoting growth. The term accessory carries with it the idea of playing a subordinate rôle, and in this sense has been employed by writers on dietetics as synonymous with condiment.[9] While we have, following Hopkins, employed this term up to the present time, we have felt that it is by no means satisfactory.

We would, therefore, suggest the desirability of discontinuing the use of the term vitamine, and the substitution of the term fat-soluble A and water-soluble B for the two classes of unknown substances concerned in inducing growth. These terms have the merit of not attributing extravagant values to these bodies, and they differentiate between the substances or groups of substances only with respect to their solubility relations, which is the only basis of differentiation at present known. These terms have the additional advantage that further letters may readily be introduced as investigation progresses, provided there proves to be more than a single representative of each class, and also that they will automatically fall into disuse when we come to possess definite knowledge of their chemical nature.

When McCollum and Davis[10] proposed the working hypothesis regarding the essential factors in the diet during growth which assumes the necessity of the two kinds of unknown substances mentioned above, they were fully aware of the apparent discrepancy between this hypothesis and the recorded experimental data which indicated that birds can be cured of polyneuritis by the employment of water extracts of certain natural foodstuffs. Such water extracts could scarcely be assumed to contain appreciable amounts of lipoidal material. In fact, nearly three years ago, one of us (M.) satisfied himself by experimental trial of the validity of the statement that a cure can be effected in this way.

In the present paper we wish to report an investigation of the relation to polyneuritis of the two classes of unknown dietary factors A and B as defined above.

[9] Howell, W. H., Textbook of Physiology, Philadelphia, 5th edition, 1913, 727, 905.

[10] McCollum and Davis, J. Biol. Chem., 1915, xxiii, 231.

Effects of Feeding Polished Rice with Butter Fat.

Bird 792A was offered a ration of rice 95 and butter fat 5 per cent from Sept. 18 to 28. It steadily lost weight and was evidently not consuming an adequate amount of food. Beginning with Sept. 28 we forcibly fed the pigeon with 20 gm. daily of the rice and butter fat mixture. On Oct. 15 it manifested the first symptoms of polyneuritis. Oct. 16, it was in a very bad condition. It was given the alcoholic extract (95 per cent) of 10 gm. of wheat embryo. Oct. 17, the bird was in a nearly normal condition.

In all, nine birds were fed with this ration and the above protocol illustrates what may be anticipated as the result of confinement to this diet.

Our observations are in accord with those of Funk and Macallum indicating that butter fat does not protect against polyneuritis birds fed polished rice and the fat.

We early became convinced that pigeons cannot be depended upon to eat enough of such experimental rations as are described in this paper, so we adopted the practice of forced feeding twice daily. This was accomplished by inserting a tube into the crop and pouring the powdered ration into it. A glass rod served to discharge the food from the tube when necessary. About 10 gm. of food were administered at each feeding. Water was given twice daily in 10 cc. doses by means of a pipette.

Effects of Feeding Polished Rice and Alcoholic Extracts of Fat-Free Wheat Embryo.

500 gm. of wheat embryo were freed from fat by ether in a continuous extractor and the residue was then extracted during 18 hours with 95 per cent alcohol also in a continuous extractor of the Caldwell type. The alcoholic extract was evaporated on 200 gm. of dextrin (Preparation 1).

Bird 1113B was offered rice for voluntary consumption from Nov. 11 to 18. After this date the bird was forcibly fed with a mixture of casein 18, salts 3.7, dextrin 78.3 per cent.

This ration will be hereafter referred to as the casein ration. The composition of the salt mixture employed was as follows:

	gm.		gm.
NaCl	0.173	CaH₄(PO₄)₂.H₂O	0.540
MgSO₄ (anhydrous)	0.266	Ca lactate, Ca(C₃H₅O₃)₂.	
NaH₂(PO₄).H₂O	0.347	5H₂O	1.300
K₂HPO₄	0.954	Fe lactate Merck	0.118

Funk[4] employed a ration of similar composition but containing butter fat, and observed that the birds were stricken wth polyneuritis after a period comparable to rice feeding.

Nov. 26, the pigeon was in the typical condition of severe polyneuritis. It was given Preparation 1 equivalent to 10 gm. of wheat embryo, morning and evening. Nov. 27, it was able to sit in a normal position, but could not use its legs. It was fed as on the 26th. Nov. 28, it was given 10 gm. of Preparation 1 and 10 gm. of the casein ration. Nov. 29, the bird could walk. We continued feeding as on Nov. 28 to Jan. 12 when the experiment was discontinued, the bird being in a normal condition.

The presence of the fat-soluble A in wheat embryo was established by McCollum and Davis.[6] They did not study the growth-promoting power of the isolated wheat embryo fat, however, and only after these experiments were well under way did we learn in another connection in feeding work with rats that the fat may be quantitatively removed from wheat embryo and yet the fat-free residue, when added to a ration of purified food materials, can induce in rats a growth curve entirely unlike those obtained with diets lacking the unknown A.[11] This led us to conclude that the recovery from polyneuritis and prolonged well-being of pigeons given alcoholic extract of fat-free wheat embryo, was not a demonstration of recovery and subsequent maintenance on a diet free from the fat-soluble A, since this is in the fat-free embryo and might be extracted with alcohol. We next sought to induce the recovery of pigeons by the administration of nearly lipoid-free preparations from other sources.

Experiments with Potato Juice.

Vedder and Clark[12] employed potato juice and found it inactive. Our experiments with potato juice gave results indicating that it possesses a moderate curative power.

Bird 1169B was forcibly fed from Nov. 14 to Dec. 4 with rice until polyneuritis was imminent. It was continued on rice and from Dec. 4 was given 12 cc. of fresh potato juice twice daily. The bird could walk very unsteadily from Dec. 8 to 18 when it so improved as to walk naturally, flew voluntarily to a perch, and appeared normal. The potato juice

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[11] In a subsequent paper from this laboratory soon to appear this point will receive full consideration.

[12] Vedder, E. B., and Clark, E., *Philippine J. Sc., B.*, 1912, vii, 423.

was continued until Jan. 12 when the forced feeding was discontinued, the bird appearing normal. The ration of rice with potato juice evaporated upon it was offered for voluntary consumption. The consumption was entirely inadequate and the bird rapidly lost weight and strength during the first week. On changing it to a diet of corn meal it regained its normal condition.

Since the pigeons invariably grow emaciated when given diets which induce polyneuritis and may fully recover with proper dietary treatment without appreciable increase in weight, together with the fact that considerable fluctuation in body weight may result from the retention of food in the crop as the time for the onset of the pathological symptoms approaches, we do not believe that the value of our records is enhanced by the inclusion of the weight records. This portion of our data is therefore omitted.

Experiments with Cabbage Juice.

Bird 862B. This bird was forcibly fed with 25 gm. of polished rice per day from Nov. 11 to Dec. 6. From Nov. 24 the crop was continually packed with food and very hard. Locomotion was distinctly impaired. On Dec. 6 and thereafter two doses of cabbage juice of 15 cc. each were administered daily—morning and evening. The crop became soft when rice and cabbage juice were both administered and on Dec. 8 the bird walked much better. It never returned to a normal condition, however, and although the regular feeding with this mixture was continued, it occasionally regurgitated food and on Dec. 19 the crop was again packed with food. Dec. 20, it was in the typical polyneuritic condition. Three doses of cabbage juice were given on this date and the bird was still alive on the morning of the 21st and very weak, but the head was not retracted. Dec. 21, five doses of cabbage juice representing a total of 180 cc. evaporated to a pasty consistency were given. The symptoms of polyneuritis were in great measure relieved, but the bird was very weak and died at 9 a.m., Dec. 22. There is no doubt that the cabbage juice contains the curative factor, but apparently in a quite low concentration.

Bird 1113 B. Forcibly fed with rice from Jan. 12 to 29 when it showed by its retracted head and inability to walk that it was in the polyneuritic state. In the morning we gave it cabbage juice (15 cc.) and 10 gm. dextrin on which 70 cc. of cabbage juice had been evaporated. In the afternoon the same dosage was administered. Jan. 30, the bird was normal in its movements and appeared cured.

Experiments with Oat Extract.

Bird 1091H was forcibly fed polished rice from Nov. 12 to Dec. 6 when symptoms of polyneuritis appeared. It was fed Dec. 6, morning and

afternoon, 12 gm. of rice on which a water extract of 24 gm. of rolled oats had been evaporated. 500 gm. of finely ground rolled oats were shaken in a shaking machine with 2 liters of water for 1 hour. The material was then centrifuged and the supernatant liquid poured off and evaporated on 500 gm. of rice. At each feeding 12 cc. of water were introduced into the crop. This treatment was thereafter continued daily until Dec. 16. The recovery was slow, but progressive, the crop remaining hard. On Dec. 12 the crop was washed out with a stream of warm water introduced through a rubber tube. The bird was apparently normal on Dec. 16 and thereafter would fly up to a perch and remain there when not disturbed. Forced feeding of this ration was continued until Jan. 19. For 3 weeks preceding the termination of the experiment this bird was very spirited and would persistently attack and annoy any other bird which was introduced into its cage, so it had to be kept isolated.

This experiment is of special interest because of the behavior of rats when fed rolled oats alone as contrasted with a mixture of rolled oats plus butter fat. When confined to rolled oats or the oat kernel freed from hulls in the laboratory, young rats steadily lose weight. On adding butter fat (5 per cent) to the oats they slowly gain in body weight and continue to exhibit a well nourished appearance for months.[18] From this we draw the conclusion that the oat kernel contains little if any of the fat-soluble A, since without its addition even maintenance is not attained, while growth proceeds when this factor is added. Since a solvent such as water, which exerts a very slight solvent action on lipoidal material, removes from oats a substance which cures polyneuritis, it points strongly to the belief that the cure is affected without the intervention of the fat-soluble A. The possibility remains, of course, that the requirement of the fat-soluble A is small for maintenance as compared with the amount necessary for growth. Since both classes of these unknown essential components of the diet are present in the vegetable world, and we have as yet no method perfected for testing for very small quantities of either A or B, this point cannot yet be definitely decided. Since, however, a pigeon can be maintained in excellent condition, as was Bird 1091H, by a mixture of 20 gm. of rice plus the water extract of 24 gm. of rolled oats per day, and growth cannot be attained with the content of the fat-soluble A in 100 per cent of rolled oats, we feel safe in asserting that the maintenance requirement of the

[18] An extended study of the dietary deficiencies of the oat kernel is now in progress in this laboratory and will be reported in a later paper.

bird for the fat-soluble A is less than the growth requirement of the rat.

There appears to be good evidence that the fat-soluble A tends strongly to remain associated with non-lipoid materials. This we have mentioned above in connection with the growth-promoting power, with respect to this particular factor, which is exhibited by wheat embryo completely extracted with ether. Further support of this idea is found in the growth curves obtained by Osborne and Mendel[14] with their protein-free milk. These curves showing normal growth over periods of 3 months or more could have been obtained only with rations which provide this dietary factor. Since they have shown that ether extracts almost nothing from protein-free milk it follows that it is difficult to free food materials from this substance.

We have endeavored to advance in the matter of determining whether water extracts of lipoid-free foodstuffs can effect a cure of polyneuritis in pigeons. To this end 500 gm. of wheat embryo were extracted exhaustively with ether and afterwards with carbon tetrachloride. The extraction with carbon tetrachloride for $22\frac{1}{2}$ hours at a good rate in a continuous extractor removed only 0.07 per cent of material from the ether-extracted embryo. The residue after exhaustion with both these solvents was extracted with water by stirring the embryo into boiling water and adding acetic acid to facilitate the complete coagulation of dissolved protein. The material was centrifuged and the clear supernatant liquid was evaporated on 500 gm. of dextrin (Preparation 2). This product had curative properties in a high degree.

Bird 910A. Fed rice Dec. 21 to Jan. 10 when it manifested typical polyneuritis. Jan. 10, it was given two doses of 5 gm. each of the casein ration and of Preparation 2. Jan. 11, p.m., the bird was slightly improved, but was in danger of dying during the night, so it was given a dose of the curative substance prepared by extracting 10 gm. of Preparation 2 with water. Jan. 12, a.m., the bird was in a normal condition. Thereafter it was given the casein ration carrying the Preparation 2 equivalent to 5 per cent of lipoid-free germ. On Jan. 16 its crop was hard and only water was given in the morning. In the evening the crop was still packed and hard and the bird was again showing incipient polyneuritis so it was given the strong solution from 10 gm. of Preparation 2. Jan. 17, a.m., the bird was again

[14] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1912, xii, 81.

normal. Thereafter it was given daily 20 gm. of the casein ration carrying Preparation 2 equivalent to 10 per cent of lipoid-free embryo. It remained normal until Jan. 23, p.m., when it again had polyneuritis. It recovered again with the strong solution, and the experiment was discontinued.

This record was duplicated in all essentials with two other pigeons, and leaves no room to doubt that the water extract of wheat germ made lipoid-free by thorough extraction with ether and with carbon tetrachloride induces a complete recovery from polyneuritis.

Experiments with Acetone Extract of Fat-Free Wheat Embryo.

For the isolation of the curative agent it is of the utmost importance to have an extensive knowledge of the solubility of the substance in all the ordinary solvents. With a view to obtaining such evidence we have administered to polyneuritic pigeons the extract of wheat embryo prepared by first removing all ether-soluble matter, and then extracting the residue at a vigorous rate in an extractor of the Caldwell type with acetone for 18 hours. The acetone-soluble material from 500 gm. of wheat embryo, which amounted to 3.2 gm., was incorporated with 396.8 gm. of dextrin (Preparation 3).

Bird 1096. Ate polished rice voluntarily from Dec. 14 to Jan. 13, when it was in incipient polyneuritis. It was given water extract of 10 gm. of Preparation 3. Jan. 14 signs of paralysis had disappeared. Gave the same water extract and rice in the morning. In the afternoon gave water only, as the crop was still hard. Jan. 15, the bird was in good condition. Fed the casein ration carrying the acetone extract of 5 per cent of wheat germ. Jan. 18, the crop was hard so we gave water only. Jan. 19, the crop was empty and the usual food given. Jan. 20, the crop was rather hard, the bird walked unsteadily. It was given 10 gm. of food in the morning; only water in the afternoon as the crop was packed. Jan. 21, the crop was hard, the bird walked unsteadily. It was given the casein ration carrying 10 per cent of acetone extract of embryo, but otherwise like that fed after Jan. 15. Jan. 22 the bird had a severe attack of polyneuritis in the morning. We gave strong water extract of 10 gm. of Preparation 3. Repeated this at noon. In the afternoon it was still very ill, so it was given another dose at 4 o'clock. At 8 p.m. it was on its feet. The dose was repeated. Jan. 23, it was nearly normal. We gave it the water extract of 10 gm. of Preparation 3 plus 5 gm. casein ration. Jan. 24, the bird was in normal condition, and the experiment was discontinued.

The above protocol is typical of three records obtained with acetone extract of wheat embryo. The curative substance is extracted by this solvent, in the course of long continued extraction.

Experiments with Benzene Extract of Fat-Free Wheat Embryo.

1 kg. of wheat embryo was extracted for 18 hours with ether and the residue afterward extracted in a Caldwell extractor for 18 hours with benzene. The benzene was evaporated on 200 gm. of dextrin. The extract amounted to 3.6 gm. (Preparation 4).

Bird 1172 from Dec. 14 was fed the casein ration. On Jan. 19 it began to regurgitate its food. Jan. 23, it had a bad attack of polyneuritis and was given a water extract of 10 gm. of Preparation 4, morning and evening. Jan. 24, it was much better, but still had symptoms of the disease. The same treatment was repeated. Jan. 25, it was nearly normal. Gave it 10 gm. of Preparation 4 plus 10 gm. of the casein ration in the morning. In the afternoon it appeared normal.

Another bird was cured after a similar history by this preparation.   •

Experiment with Ethyl Acetate Extract of Fat-Free Wheat Embryo.

400 gm. of wheat germ were extracted with ether during 48 hours. The residue was extracted for 14 hours with ethyl acetate and the ethyl acetate evaporated on 400 gm. of dextrin (Preparation 5).

Bird 968B was forcibly fed with rice from Dec. 14 to Jan. 31 when it had a severe attack of polyneuritis. It was given a water extract of Preparation 5, equivalent to 10 gm. of wheat embryo, in the morning. In the afternoon of Jan. 31 it could walk fairly well, and the dose was repeated. Feb. 1, the bird was in a normal condition.

The results of these experiments help in clarifying the apparent lack of harmony in the dietary requirements of the mammal during growth, and the relief of polyneuritis in birds. Both the fat-soluble A and the water-soluble B must be present in the diet of the mammal during growth. The absence of the former leads to failure to grow, to emaciation, and to a pronounced inflammation of the eyes, all of which conditions, provided the other factors in

the ration are adequate,[15] are promptly relieved by the administration of fats of the group which possess the peculiar properties of butter fat. No matter how generous may be the supply of the fat-soluble A, failure of nutrition will result, provided an adequate amount of the water-soluble B is not also furnished by the diet.[16] Judging from the appearance of serious nutritional disturbances ending in death, which result from a shortage of the fat-soluble A, and the emaciation, weakness, and death which follow restriction to a diet inadequate in its content of the water-soluble B, *it seems certain that both these classes of unknown dietary constituents are essential for maintenance as well as for growth.*

SUMMARY OF CONCLUSIONS.

1. The data presented in this paper show conclusively, we believe, that in the production of polyneuritis in birds by exclusive rice feeding or exclusive feeding of a ration made up of purified foodstuffs, the degeneration of the nerve cells is the specific result of a lack of the water-soluble B. *The fat-soluble A appears to be dispensable when maintenance alone is involved, for a somewhat longer period than is the factor B.*

We base this last assertion upon the fact that pigeons can be brought into the polyneuritic state by feeding a diet free from both the essential factors A and B, and can be completely cured and maintained in a normal condition for at least 35 days on the same diet which brought on the disease, plus the water extract of a foodstuff (rolled oats) on which rats cannot grow without the addition of butter fat, but on which they do grow when the latter is added. Confirmatory evidence is also offered in our success in inducing relief from polyneuritis in birds, by treatment with water extract of wheat embryo, previously rendered lipoid-free by exhaustion with ether and carbon tetrachloride successively, and also in our success in curing birds with such substances as cabbage or potato juice, both of which are practically free from lipoids. The results with these lipoid-free products, we present

[15] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 180. Osborne and Mendel, *J. Biol. Chem.*, 1913, xvi, 431.

[16] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181, 199, Chart 5, and 221, Chart 33.

with a full appreciation of the fact that the fat-soluble A is found in considerable amount in such natural foods as wheat germ even after practically complete removal of the lipoids.

2. We also present experimental data showing that acetone, benzene, and ethyl acetate extract from wheat embryo, previously rendered fat-free by extraction with ether, the substance which relieves the symptoms of polyneuritis in pigeons.

A COLORIMETRIC METHOD FOR THE ESTIMATION OF AMINO-ACID α-NITROGEN. II.

APPLICATION TO THE HYDROLYSIS OF PROTEINS BY PANCREATIC ENZYMES.

By VICTOR JOHN HARDING and REGINALD M. MacLEAN.

(*From the Biochemical Laboratory, McGill University, Montreal.*)

In the first communication on this subject[1] the authors showed that the interaction of amino-acids and triketohydrindene hydrate (ninhydrin reaction) in presence of pyridine could be used as a means of estimating amino-acid α-nitrogen colorimetrically. The amount of blue coloring matter (ammonium salt of diketohydrin-dylidene-diketohydrindamine) formed is proportional to the amount of amino-acid α-nitrogen when the latter is between the limits of 0.05 mg. and 0.005 mg. per cc. of liquid to be estimated. It was claimed that the method could be used as a means of following protein hydrolysis and this present paper contains the evidence in support of such a belief.

We have also subjected to criticism and reexamination the matter of our previous paper where it affects the method for our colorimetric determination, and a year's experience with the process has shown us one or two points which require amplification.

The Standard Alanine Solution.—The standard alanine solution is made as previously directed by dissolving 0.3178 gm. of freshly crystallized alanine in water and making up the volume to 1 liter. Its stability varies a little. Thus one solution was found to be stable for a period of 3 months while a second only gave about 95 per cent of the theoretical amount of standard color at the end of that time. In consequence we have found it advisable to check the stability of our standard alanine by comparing it from time to time with a freshly prepared solution.

The Standard Color.—We have made several further attempts to prepare a permanent standard color, but without success. The

[1] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1915, xx, 217.

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direct preparation of the ammonium salt of diketohydrindylidene-diketohydrindamine by the interaction of a mixture of ammonium acetate and carbonate upon hydrindantin[2] is anything but quantitative, and conditions have not been found under which the blue coloring matter is absolutely stable. We have also attempted to prepare artificial standards from mixtures of various dyestuffs and coloring matters. The only partially successful experiment was a mixture of phenolphthalein and thymolphthalein in alkaline solution. This matched the standard alanine color perfectly, but possessed no advantages over it, as it was even less stable. The standard color is prepared as previously directed. 1 cc. of 10 per cent aqueous solution of pyridine may replace the 0.5 cc. recommended, if it is so wished.

The stability of the standard color seems to vary. Our first statement is undoubtedly incorrect, and we can only ascribe the error to some confusion of notes. The minimum time of stability of the standard color which we have observed is 3 hours; the maximum is 12. Hence we cannot recommend the use of a standard color which has been prepared over a period longer than 3 hours.

The production of the color in the standard, and hence in the determinations, is in all probability a quantitative one in the absolute sense. Thus it cannot be increased by the use of further excess of either pyridine or triketohydrindene hydrate.

1 cc. standard alanine.		Colorimeter reading.
1 cc. pyridine. Concentration.	1 cc. ninhydrin. Concentration.	
per cent	*per cent*	
5	1	1.07
10	1	1.00
10	2	1.01
15	1	0.98
25	1	1.00

The use of 1 cc. of 5 per cent aqueous solution of pyridine, however, gives too low a result. This is interesting as 0.5 cc. of 10 per cent aqueous solution of pyridine gives the maximal value.

[2] Ruhemann, S., *J. Chem. Soc.*, 1911, xcix, 1486.

Maximal and Minimal Amounts of Amino-Acid α-Nitrogen Determinable by the Method.—These two points were fixed at 0.05 mg. and 0.005 mg. nitrogen per cc. Judging by our experience with the various proteins cited in this paper, the method gives accurate results with 0.08 mg. of amino-acid α-nitrogen per 1 cc. When dealing with the mixtures of the amino-acids it appears quite a safe procedure to estimate the α-nitrogen in a higher concentration than is possible with some of them by themselves, and this is, of course, to be expected. Even this value is probably too low, but we prefer to err on the safe side. The lower limit, however, is perhaps too low for practical convenience in this field of work. It is possible to obtain accurate results at that concentration of nitrogen, but they seem to be extremely susceptible to error, and if it is possible, we would not advise the use of a limit lower than 0.01 mg. amino-acid α-nitrogen per cc. Even then great care must be taken in the determination.

The Case of Cystine.—As was pointed out in the previous communication, cystine is the one amino-acid among those examined which fails to give quantitative results. Furthermore we have not been able to devise any modification whereby this amino-acid can be estimated by our colorimetric process. The colors produced are always very red and very low in amount, due to the formation of either excessive amounts of hydrindantin or sulfur derivatives. Judged by the Van Slyke method as standard, however, it does not seem to interfere in the estimation of the amino-acid α-nitrogen in the majority of hydrolyzed proteins, thus bearing out our contention of the previous paper.

Method of Estimation of Amino-Acid α-Nitrogen by the Colorimetric Process.

1 cc. of the solution to be estimated, containing between 0.08 and 0.01 mg. of amino-acid α-nitrogen and neutral to phenolphthalein, is mixed with 1 cc. of 10 per cent aqueous solution of pure pyridine, and 1 cc. of a freshly prepared 2 per cent solution of triketohydrindene hydrate (ninhydrin), in a test-tube, and stoppered lightly with an absorbent cotton tampon. The test-tube is placed in a rapidly boiling constant level water bath for a period of 20 minutes, then removed, cooled by immersion in cold water, and diluted to a suitable volume, which must be arranged so that

the colorimetric readings do not differ greatly from the reading of the standard color. The comparison is made in a Duboscq colorimeter, placed in a dark room, and illuminated as described in our previous paper (page 221), and too much stress cannot be laid on this point.

The standard color is prepared by similarly heating 1 cc. of standard alanine solution, 1 cc. of 10 per cent aqueous pyridine, and 1 cc. of 1 per cent solution of triketohydrindene hydrate, for a period of 20 minutes, cooling, and diluting to 100 cc. In estimating solutions containing small amounts of amino-acid α-nitrogen (0.03–0.01 mg. per cc.), it is advisable to use a standard of half the ordinary strength (1 cc. = 0.025 mg. of nitrogen) and dilute the color to 50 cc. It is important that the water bath used in heating the solutions shall be large and of constant level, otherwise the heating may become uneven and irregular results be obtained.

Application of the Method to the Study of the Hydrolysis of Proteins by Pancreatic Enzymes.

In order to determine experimentally the application of our colorimetric method to a study of proteoclasis, we have followed by means of it the hydrolysis of various types of proteins by pancreatic enzymes, and compared the results with those obtained by the Sörensen and Van Slyke methods. The use of the pancreatic enzymes, as proteoclastic agents, gives results which are not complicated by other factors, such as high acidity or the production of ammonia,[3] and thus makes all determinations simpler and less subject to error. The hydrolysis was allowed to proceed for 3 or 4 days in presence of toluene until the amino-acid α-nitrogen value had become almost constant. It is to be noted, however, that in no case did the values ever become absolutely constant, though the rate of hydrolysis became very small, yet it never entirely ceased; even at the end of 216 hours the hydrolysis of casein was still proceeding.

The proteins used in these experiments were serum albumin and globulin (Schuchardt), gluten (Schuchardt), fibrin (ox blood), gelatin, casein (purified), nucleoprotein (Schuchardt), peptone (Witte),

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[3] Andersen, A. C., *Biochem. Z.*, 1915, lxx, 344.

and peptone *e carne* (Schuchardt). They were used in solutions of approximately 0.5 per cent, and to 100 cc. of the protein solution were added 1 cc. of the pancreas preparation, 1 cc. of 0.5 per cent sodium carbonate solution, and 2 cc. of toluene. Several such solutions were made and all incubated together at 38–39°C.

The enzyme solutions used were two in number. The first was a preparation of the pancreas by Fairchild Brothers and Forster, and sold under the name of *holadin*, and was used in either a 0.2 or a 1 per cent solution. This preparation is supposed to contain trypsin. We found, however, that although it was active towards casein, gelatin, and peptones, it was entirely inactive towards serum albumin and globulin, thus corresponding in its properties to erepsin rather than to trypsin. We later found that its proteoclastic powers could be extended to fibrin and to gluten. There is undoubtedly, even yet, much uncertainty and confusion existing about the activities of these two classes of proteoclastic enzymes, and the age of our sample may have been the cause of our result. Nevertheless, as the enzyme was inactive towards albumin and globulin, we prefer to class it as the pancreatic erepsin discovered by Vernon[4] rather than as trypsin. It did not belong to the autolytic class of enzymes producing large quantities of ammonia. We examined this point experimentally and found the following amounts of ammonia produced by the action of this enzyme on a 0.5 per cent solution of protein in 100 hours, and compared them with the amounts of amino-acid α-nitrogen.

100 cc. of the protein solution (0.5 per cent).	Ammonia nitrogen.	Amino-acid α-nitrogen.
	mg.	*mg.*
Casein................................	0.67	22.7
Peptone (Witte)	0.28	24.3
Peptone *e carne*...................	0.44	17.0

The small amounts of ammonia are thus insignificant compared with the amounts of amino-acid α-nitrogen.

The second enzyme preparation was an alcoholic extract of pig's pancreas prepared according to the directions given by Cole.[5] This agreed in its proteoclastic properties with trypsin.

[4] Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.

[5] Cole, S. W., Practical Physiological Chemistry, Cambridge, 1913, 3rd edition, 94.

At the end of stated intervals the flasks containing the hydrolysis experiments were removed from the thermostat, boiled for about 15 seconds, and the amino-acid α-nitrogen content was determined by the three aforementioned methods.

The colorimetric method was applied, as stated in this paper, directly to the solution. The alkalinity caused by the presence of the sodium carbonate was found to be too small to affect the results.

In the Van Slyke method we used the micro form of the apparatus.[6] Care was taken in the standardization of the apparatus and the use of reagents.

To apply the Sörensen method 20 cc. of solution neutral to phenolphthalein were taken, excess of neutral formaldehyde was added, and then titrated with 0.1 n NaOH, until a deep pink solution resulted, the color of which was permanent a full 5 minutes.

The general results can best be seen by an inspection of the curves shown in Figs. 1 to 8.
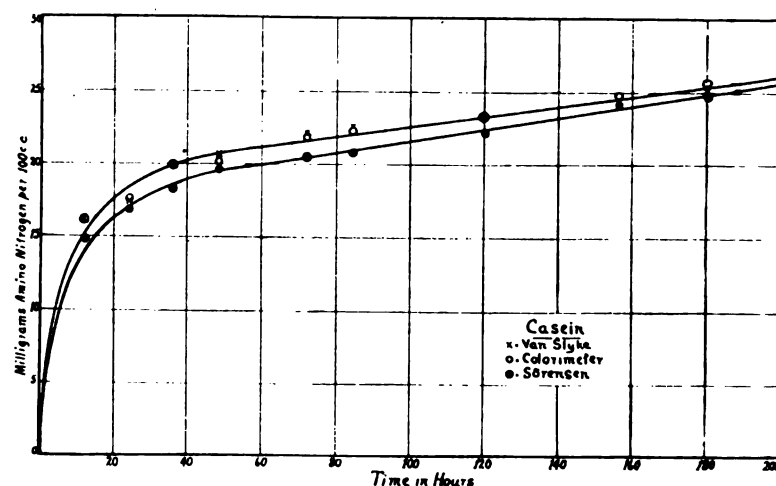
FIG. 1.

[6] Van Slyke, D. D., J. Biol. Chem., 1913–14, xvi, 121.

Fig. 2.

Fig. 3.

Fig. 4.

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FIG. 5.

FIG. 6.

FIG. 7.

510
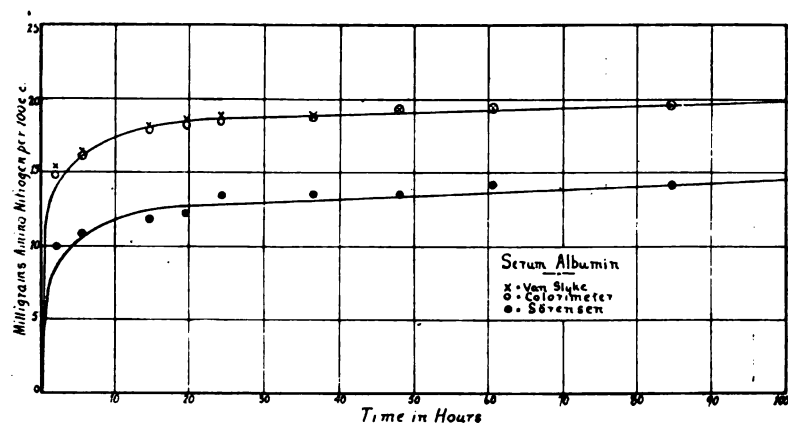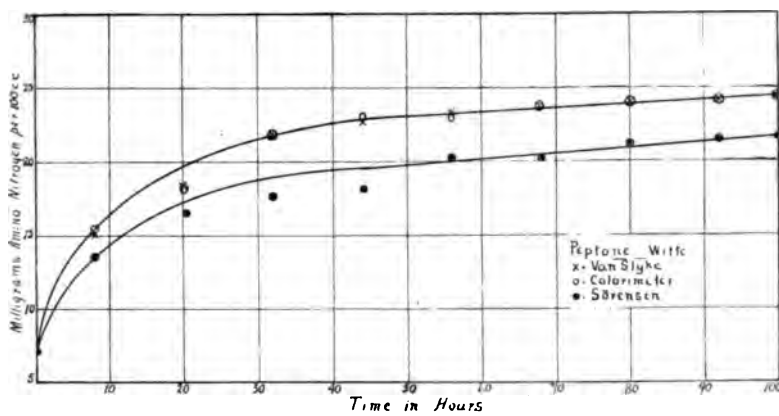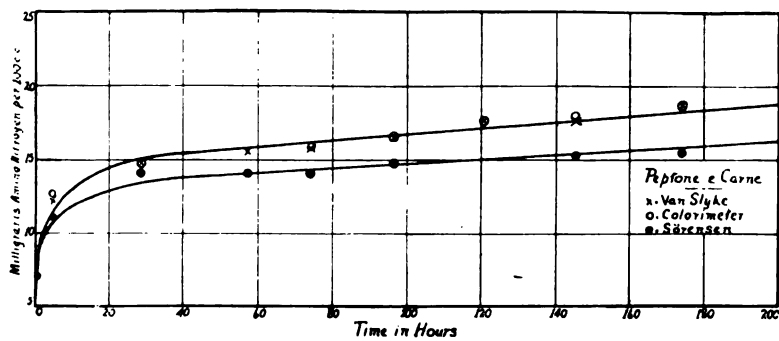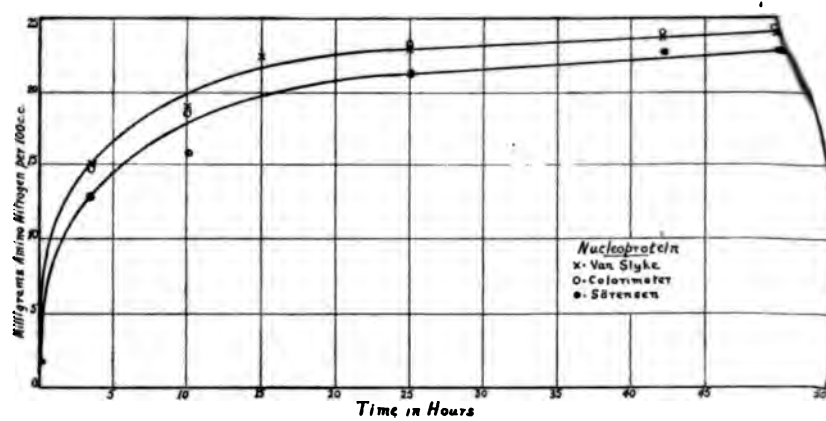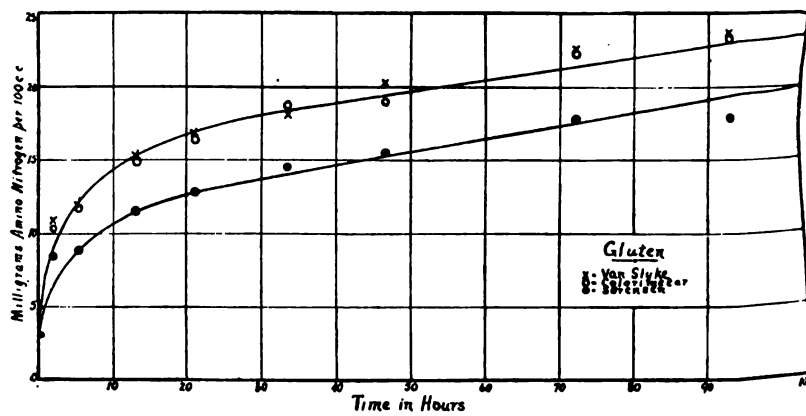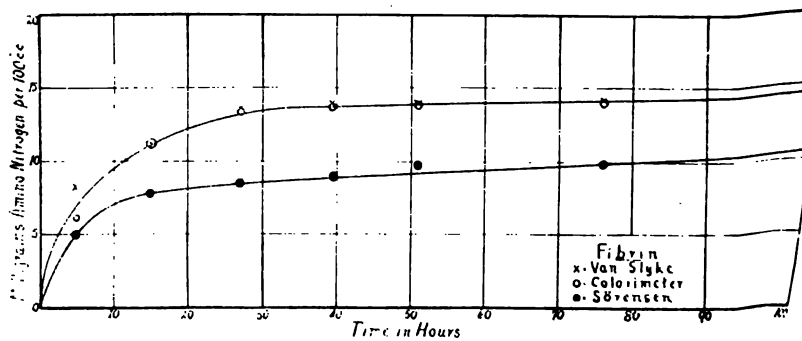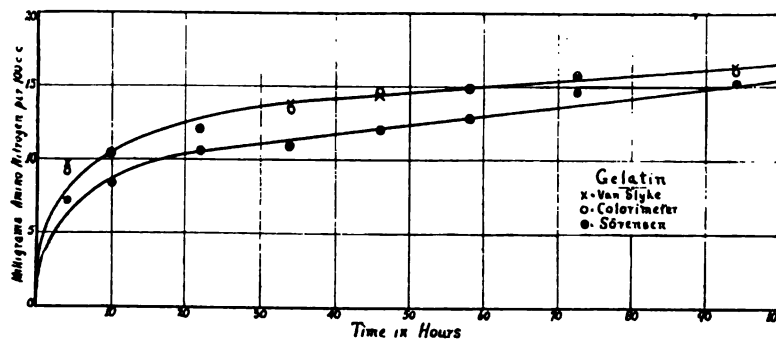
FIG. 8.

DISCUSSION OF RESULTS.

The results of the experiments show quite clearly that the colorimetric method is applicable to a study of proteoclasis. The striking agreement of the method with the Van Slyke method and the general agreement of the two with the Sörensen method show that it estimates quantitatively the hydrolysis of the peptide chain. Qualitatively all three methods give the same information, and can be used to determine the presence of peptide groupings in an unknown substance or mixture, or to detect the presence of a proteoclastic enzyme when acting on a peptide substrate. The agreement of the colorimetric and gasometric methods is to be expected, for both give theoretical results with aqueous solutions of pure amino-acids, except in the case of glycocoll with the gasometric method, and cystine with both methods. Both methods give identical results when applied to peptone solutions obtained from various sources. A reference to the experimental figures, however, will show that with some native proteins and during the early stages of the hydrolysis of these proteins the two methods do not give identical results. This is shown in the following table, very clearly in the case of the mixture of serum albumin and globulin, and of fibrin.

Protein.	Time.	N₂ per cc.	
		Van Slyke.	Colorimeter.
	hrs.	*mg.*	*mg.*
Serum albumin and globulin.......	0.0	0.0612	0.0200
Gluten............................	0.0	0.0313	0.0293
Fibrin............................	5.0	0.0833	0.0602
Gelatin.......:...................	1.0	0.0352	0.0320
Casein...........................	0.5	0.0386	0.0384
Nucleoprotein....................	0.0	0.0174	0.0176
Witte peptone...................	0.0	0.0734	0.0719
Peptone *e carne*.................	0.0	0.0777	0.0807

As the hydrolysis proceeds, however, the figures begin to come into agreement and at the end of 15 hours they read:

	Van Slyke.	Colorimeter.
	mg.	*mg.*
Serum albumin and globulin........	0.1812	0.1782
Fibrin............................	0.1110	0.1111

The significance of the discrepancy is not apparent. It cannot be due to the partial interaction of the ω-amino group of lysine with nitrous acid and not with triketohydrindene hydrate, otherwise the same discrepancy would show itself with casein and peptone (Witte). We are forced for the present to ascribe it to some physical characteristic of the proteins mentioned. The agreement of the results of the two methods with the remaining proteins in the very early stages of hydrolysis and with the peptones makes it extremely probable that the colorimetric process estimates the amino group α to a carboxyl group, even when the latter is bound in a peptide linking, as amide nitrogen gives no ninhydrin reaction. The point, however, requires fuller experimental investigation with purified peptones and synthetic polypeptides, etc.

On comparing the three methods of following protein hydrolysis in very dilute solution, and where the results are not complicated by the presence of large amounts of ammonia, it is evident that the colorimetric or the Van Slyke methods are to be preferred. The Sörensen method, however, is much the simplest to

carry out, though its range of application does not allow it to be used in extremely dilute solutions of proteins.[7]   We should, however, mention in this connection a recent paper by Clementi,[8] who, by an improved and accurately calibrated burette, has made the Sörensen method sensitive with solutions containing less than 1 mg. of amino-acid per 1 cc.   The most sensitive method is undoubtedly the colorimetric method, and it is also relatively simple in manipulation.   It is inapplicable, however, in strongly acid or alkaline solutions, and will probably find its greatest usefulness in the study of protein hydrolysis in neutral and faintly alkaline media.   The gasometric method, owing to its susceptibility to correction, for the nitrogen of amino groups other than the α-amino group of amino-acids, is the most accurate of the three. The ninhydrin reaction is also given by a number of amines;[9] but it will be shown in a later paper from this laboratory that these are capable of classification, and fall into one or two well defined groups.

SUMMARY.

The colorimetric method for the estimation of amino-acid α-nitrogen can be used to follow the hydrolysis of proteins by pancreatic enzymes.   The results agree with those obtained by the Van Slyke method.

EXPERIMENTAL.

Casein.

The casein employed in the following digestion was purified according to the method of Robertson.[10]   5.6 gm. of this pure casein were dissolved in 44.8 cc. 0.1 N NaOH in pursuance of Walters'[11] directions for preparing basic sodium caseinate (0.4 per cent).   To this were added 300 cc. distilled water and, after boiling and filtering, the clear filtrate was made up to 1,400 cc.

[7] Potter, R. S., and Snyder, R. S., *J. Ind. and Eng. Chem.*, 1915, vii, 1049.

[8] Clementi, A., *Atti Accad. Lincei*, 1915, series 5, xxiv, 51, 102.

[9] Neuberg, C., *Biochem. Z.*, 1913, lvi, 500.

[10] Robertson, T. B., *J. Phys. Chem.*, 1910, xiv, 534.

[11] Walters, E. H., *J. Biol. Chem.*, 1912, xi, 269.

100 cc. protein solution, 1 cc. 0.5 per cent Na_2CO_3, 1 cc. 1 per cent holadin solution, and 2 cc. toluene were used in each experiment.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0.5	0.0386	0.0297	0.0384
12	0.1628	0.1484	0.1638
24	0.1748	0.1696	0.1754
36	0.1991	0.1838	0.2000
48	0.2068	0.1987	0.2082
72	0.2211	0.2050	0.2180
84	0.2257	0.2121	0.2222
120	0.2329	0.2263	0.2326
156	0.2440	0.2403	0.2462
180	0.2510	0.2474	0.2564
216	0.2657	0.2615	0.2632

Serum Albumin and Globulin.

In the endeavor to prepare a 0.5 per cent solution, 6 gm. were dissolved in dilute NaCl solution and filtered. The clear filtrate was diluted to 1,200 cc. and employed as follows: 100 cc. protein solution, 1 cc. 0.5 per cent Na_2CO_3, 1 cc. pancreatic extract, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke. [*]	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0	0.0612	0.0282	0.0200
2	0.1538	0.0991	0.1471
5.5	0.1638	0.1061	0.1622
14.5	0.1812	0.1169	0.1786
19.5	0.1841	0.1204	0.1818
24	0.1880	0.1346	0.1832
36.5	0.1880	0.1346	0.1872
48	0.1915	0.1346	0.1922
60.5	0.1922	0.1417	0.1930
84.5	0.1950	0.1417	0.1952
114.5	0.1997	0.1558	0.1976

Peptone (Witte).

A 0.5 per cent solution of peptone (Witte) was made by boiling and filtering off a slight insoluble residue. To 100 cc. portions of this solution were added: 1 cc. 0.5 per cent Na_2CO_3, 1 cc. 0.2 per cent holadin solution, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	mg.	mg.	mg.
0	0.0734	0.0707	0.0719
8	0.1511	0.1357	0.1538
20.5	0.1518	0.1640	0.1801
32	0.2165	0.1753	0.2150
44	0.2267	0.1809	0.2273
56	0.2332	0.2036	0.2296
68	0.2379	0.2036	0.2381
80	0.2396	0.2112	0.2409
92	0.2417	0.2149	0.2424
104	0.2441	0.2149	0.2439
128	0.2451	0.2205	0.2438
140	0.2467	0.2262	0.2462

Peptone e Carne.

A 0.5 per cent solution of peptone e carne was prepared by boiling and filtering off the slight insoluble residue. To 100 cc. portions of this solution were added: 1 cc. 0.5 per cent Na_2CO_3, 1 cc. 0.2 per cent holadin solution, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	mg.	mg.	mg.
0	0.0773	0.0707	0.0807
5	0.1232	0.1201	0.1250
29	0.1477	0.1414	0.1471
57	0.1552	0.1414	0.1666
74	0.1569	0.1414	0.1586
96	0.1694	0.1484	0.1666
120.5	0.1749		0.1754
145	0.1762	0.1520	0.1786
174	0.1860	0.1555	0.1852
218.5	0.1922	0.1555	0.1952

Nucleoprotein.

2.5 gm. nuclein (Schuchardt) were dissolved in 60 cc. 0.5 per cent Na_2CO_3 by boiling. After filtering, a residue of 0.2370 gm. remained, and the filtrate was diluted to 500 cc. 50 cc. samples were treated with 0.5 cc. pancreatic extract and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0	0.0174	0.0177	0.0176
3.5	0.1500	0.1275	0.1275
10.25	0.1895	0.1558	0.1851
15	0.2244		
25.25	0.2297	0.2125	0.2322
42.25	0.2371	0.2267	0.2388
55.25	0.2397	0.2267	0.2418

Gluten.

2.5 gm. gluten (Schuchardt) were dissolved in 60 cc. 0.5 per cent Na_2CO_3 at ordinary temperature, and, after filtering off a slight insoluble residue, made up to 500 cc. volume. 50 cc. portions of this solution were treated with 0.5 cc. pancreatic extract and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0	0.0313	0.0283	0.0293
2	0.1091	0.0850	0.1020
5.5	0.1182	0.0855	0.1150
13	0.1536	0.1154	0.1492
21	0.1673	0.1275	0.1640
33.5	0.1803	0.1452	0.1886
46.5	0.2036	0.1558	0.1899
72	0.2260	0.1771	0.2238
93	0.2390	0.1771	0.2343
100	0.2421	0.1842	0.2418

Fibrin.

The fibrin used in this digestion was obtained from ox blood. After removing a large excess of moisture by filter paper, analysis proved the presence of 46.98 per cent H_2O. To 100 cc. portions of distilled water were added: 0.5 gm. fibrin, 1 cc. pancreatic extract, 1 cc. 0.5 per cent Na_2CO_3, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
5	0.0833	0.0495	0.0602
15	0.1110	0.0777	0.1111
27	0.1356	0.0848	0.1338
39.5	0.1389	0.0919	0.1374
51	0.1392	0.0989	0.1388
76	0.1416	0.0989	0.1408
99.5	0.1423	0.1060	0.1424
124	0.1469	0.1131	0.1444
148.5	0.1477	0.1133	0.1471

Gelatin.

6 gm. pure sheet gelatin (isinglass) were dissolved in boiling water and, after filtering, diluted to 1,200 cc. To 100 cc. portions of this solution were added: 1 cc. 0.5 per cent Na_2CO_3, 1 cc. 1 per cent holadin solution, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
1	0.0352	0.0245	0.0320
4	0.0986	0.0707	0.0921
10	0.1031	0.0848	0.1021
22	0.1204	0.1060	0.1212
34	0.1373	0.1095	0.1351
46	0.1440	0.1201	0.1462
58	0.1489	0.1272	0.1497
72.5	0.1561	0.1484	0.1562
94	0.1636	0.1520	0.1614
119.5	0.1698	0.1520	0.1666
142	0.1725	0.1555	0.1708

IMPROVED GAS CHAIN METHODS OF DETERMINING HYDROGEN ION CONCENTRATION IN BLOOD.

By J. F. McCLENDON.

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The Gas Chain Method for Blood.

The most accurate determinations of the hydrogen ion concentration of human blood seem to be those of Hasselbalch on defibrinated reduced blood resaturated with CO_2 at alveolar tension. The objections to his method are first, the difficulties of manipulation; second, the time required to reduce the blood; and third, the error due to the deterioration of electrode and blood during reduction.[1]

In order to avoid loss of CO_2 and of time in the reduction of blood, I devised an electrode that could be filled directly from the blood vessel.[2] But in order to determine the buffer value of blood and to avoid the use of the expensive hirudin, I have modified it as follows.

Fig. 1. Hollow needle, 7 cm. long and 0.7 mm. bore.

The blood is drawn from the vein by means of the hollow needle (Fig. 1), of 0.7 mm. bore and 7 cm. long, connected with the 12 cc. defibrinating tube (Fig. 2) by means of a short piece of strong rubber tubing, in such a manner that all of the blood that is exposed to the air overflows out of the tube. A piece of glass tubing is connected to the open end of the defibrinating tube by means of rubber tubing. When the defibrinating tube is full the two rubber tubes are closed with pinch-cocks or Langenbeck

[1] Hasselbalch, K. A., and Gammeltoft, S. A., *Biochem. Z.*, 1915, lxviii, 235, 236.

[2] McClendon, J. F., *Am. J. Physiol.*, 1915, xxxviii, 181.

clips and the blood is defibrinated with the lead ball by shaking the tube. As the blood cools and contracts the clip is opened from time to time in order to allow blood to return from the overflow tube and thus prevent the escape of gas from the blood, due to diminished pressure.

Fig. 2. 12 cc. defibrinating tube.

When defibrination is complete the closure of the rubber tubes is secured by folding them back over the nipples of the defibrinating tube and wrapping them with wire or cord. The defibrinating tube is then placed in the centrifuge, balanced with a similar tube, and the fibrin and corpuscles are precipitated. When it is removed from the centrifuge the wires are replaced by clips. The lower rubber tube is connected to a long rubber tube and funnel filled with mercury in such a manner that no air is admitted. The upper rubber tube is connected with the left end of the electrode of 5 cc. capacity shown in Fig. 3. By opening the cocks and raising the funnel, the electrode is filled with serum in such a manner that all of the serum that comes in contact with the air overflows through the stop-cock into the overflow tube.

A small bubble (0.1 cc.) of pure hydrogen (or $H_2 + CO_2$) is admitted into the electrode and both ends of the latter are closed. The electrode is shaken until the bubble comes to equilibrium with the serum as regards CO_2 tension, when the bubble is allowed to rise until it completely surrounds the platinum foil. The elec-trode is then set up and allowed to come to the temperature of the room. It is connected to the electrometer by means of a copper wire hook that engages a hook on the end of the plati-num foil. The copper wire is long and coiled in a close spiral to reduce its stiffness, and is small enough so that its weight does not straighten out the hook on the platinum. If copper and platinum are bright and dry the contact is usually good enough to get the zero point with the electrometer, and if this is not the case it is immediately detected by failure of the electrometer to show a sharp zero point on the potentiometer wire. The over-flow tube is connected with the saturated KCl solution into which the calomel electrode dips, by a piece of absorbent cord. The

stop-cock is not greased and need not be opened while taking a reading, but must be opened momentarily immediately before the reading in order to equalize the pressure of the hydrogen, which has been reduced by partial combination with oxygen dissolved in the serum.
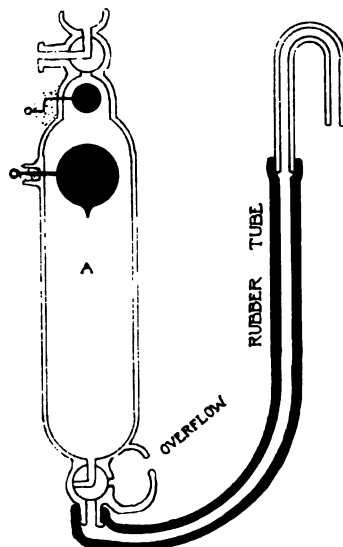
FIG. 3. Hydrogen electrode, actual size. The stop-cocks are bored at right angles so that the connecting tubes may be rinsed out through the extra openings in the stop-cocks. The lower cock is lubricated with KCl solution before filling the electrode, and its bore may be filled with the same solution and turned as in the figure if it is desired to have a wider surface of contact between this solution and the electrode contents. The two discs are gold or gold plated platinum and are platinized. In case CO_2 loss is to be avoided the upper one is used, but with known constant CO_2 pressure, the lower one is used and the H_2—CO_2 mixture forced in until only the point of the disc touches the liquid. The rubber connection filled with KCl solution allows mechanical inversion of the electrode about the axis, A, without disconnection from the potentiometer (thus replacing the absorbent cord).

A succession of readings are taken until a maximum difference of potential is obtained and remains constant 5 minutes, which denotes that all of the oxygen contained in the hydrogen has disappeared. If it is not certain that CO_2 equilibrium was established the electrode must be shaken again, after which some time

will be required to reduce the additional oxygen that has been brought up from the lower part of the electrode. Usually 200 inversions of the electrode are sufficient for CO_2 equilibrium and 10 minutes sufficient for disappearance of the oxygen.

After the above procedure the serum has the same hydrogen ion concentration as the blood in the vein except for temperature change and the loss of CO_2 into the hydrogen. By reducing the ratio of hydrogen to serum the loss of CO_2 may be made small. It may be still further reduced by using hydrogen containing 5 per cent CO_2.

The platinum foil should be 0.02–0.05 mm. thick, or if a wire is used, the part inside the electrode vessel should be beaten thin and thoroughly cleaned.

The electrodes are cleaned with potassium bichromate in H_2SO_4, are replatinized for about 30 seconds, and are tested with a standard phosphate mixture. Ten freshly platinized electrodes are tested with it and unless the majority of them agree to a millivolt the determination is repeated.

The rubber tube for the defibrination apparatus must be very strong and renewed each time. It is well to attach it with rubber cement and wire before the experiment.

The Determination of the CO_2 Pressure on the Same Sample.

Having once determined the C_H of the serum, which is the same as that of the blood in the vein, the CO_2 pressure of the sample may be approximated by exposing it to a series of CO_2 pressures and noting under which the C_H is nearest to the original value.

The CO_2 and H_2O are mixed in a tonometer. For this purpose the tonometer is made like a Hempel burette, except that on one end is wide and the other constricted and finely graduated from 0 to 10 cc. The tonometer is set up vertically with a mercury funnel and a 1 cm. vertical glass tube used as a sight in leveling the mercury surfaces. When the mercury in the funnel, tonometer, and 1 cm. tube is on a level the amount of gas introduced may be read on the scale. The tubes leading from the CO_2 and H_2 generators are connected with side tubes several meters long, filled with the respective gases in order to maintain them at atmospheric pressure while being introduced into the tonometer.

The serum is transferred to the tonometer and rotated 15 minutes by a rubber band that passes over it and an axle. The serum is returned to the electrode, a bubble of the same gas mixture added, and the reading taken. If care is taken in this manipulation, no oxygen is admitted and equilibrium is attained more quickly than for the first reading, sometimes in 2 minutes. The next lower per cent CO_2 is now used, and so through the series. The results may be compared with the alveolar CO_2 pressure.

Knowing the alveolar CO_2 tension, one can determine the P_H of arterial blood. We find it the same as venous blood within the limits of error of a single determination (without averaging duplicates). By making both electrodes the same size, duplicate determinations may be made in the same electrode vessel. If the volume of pure hydrogen is increased to 1 cc. the maximum error is 1 millivolt.

It is claimed by Hasselbalch that serum is more alkaline than blood. Hasselbalch admits that adding oxygen to blood makes it read less alkaline. Evidently oxygen acts by combining with the hydrogen in the platinum black. Serum reads more alkaline than blood because it contains less oxygen, but the reading is more nearly the true reaction of the blood. In order to decrease the danger of introducing oxygen the electrode is filled with hydrogen before use.

The Buffer Value of Serum.

Since the buffer value of a fluid means its resistance to change of reaction by the addition of acids or bases, the change of reaction on the addition or removal of CO_2 gives an index of the buffer value. It is only necessary to determine the C_H of the serum at two known CO_2 pressures to obtain an index of the buffer value. It would be convenient to have some standard of pressure, and 10 per cent and 3 per cent of an atmosphere are suggested. If the CO_2 pressure of the serum has been determined by the above method, these data are already at hand; if not, the rule should be always to begin with the higher pressure.

Thermoregulation.

Owing to the inconvenience of small thermostats, the labo—oratory was kept within 0.3° of 22° by means of an electric fan and the apparatus shown in Fig. 4. The essentials of a good system are low heat capacity of regulator and heater and rapid conduction. The regulator is made of a strip of invar and brass welded together (combined thickness, 1 mm.). The heater is bare nichrome wire strung through the air.
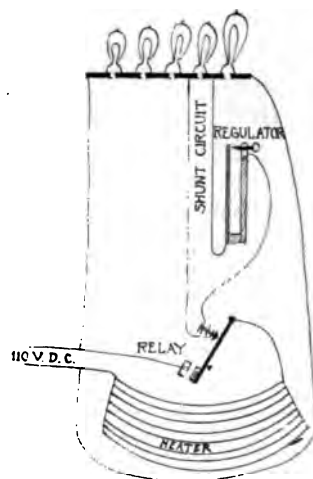
Fig. 4. Thermoregulator.

Table for Converting Millivolts to Hydrogen Ion Concentration.

Since the table by Schmidt[3] is for only one temperature, 18°, the range of 18 30° was selected in preparing the graph in Fig. 5. In 1908 the International Electrical Congress established the international ohm and fixed the ampere as the current that deposits 0.001118 gm. Ag per second. Taking the atomic weight of Ag as 107.88, the Faraday or electrical equivalent is 96,494 coulombs. The value 96,500 has come into use. If these new data are used exclusively, the formula for C_H is $P_H = - \log C_H = \dfrac{E. M. F.}{0.0001984 \ T}$

[3] Schmidt, C. L. A., Univ. California Publications, Physiology, 1905-10. iii, 104.

which is nearly the same as the old formula. The value of the volt was still uncertain, however, until the International Committee, at Washington, 1910, standardized the method of washing the cathode of the silver coulometer, and fixed the E.M.F. of the International Standard Weston Cell at 1.0183 at 20°. According to Rosa,[4] it is more nearly 1.01827, but this difference is less than the individual variations of cells. It was pointed out by Wolff[5] that no exactly correct formula for the temperature effect on the E.M.F. has been devised, and that from 2 to 24 hours are necessary for equilibrium after an abrupt change of temperature. From Wolff's formula, which is more accurate than previous formulas, it may be seen that the change in E.M.F. per degree is more nearly 0.04 millivolt than 0.038 as given by Jaeger and Wachsmuth. Where temperature control is not adequate, it might seem safer to use a cell from the Weston Company, saturated at 4°, E.M.F. = 1.0186, independent of temperature.

There is considerable difference in the correction for calomel electrodes as applied by different writers. G. N. Lewis[6] uses a value 8 millivolts different from that used by other investigators for the normal calomel electrode. He bases this value on a calculation of the dissociation of the acid used in the normal hydrogen electrode from thermodynamical data, which makes the dissociation of strong acids more nearly obey the law of mass action. Since so much work is based on the calculation of C_H from electrical conductivity, it might seem safer at present to continue this practice.

Although the saturated KCl calomel electrode of Michaelis[7] is the most convenient, if work is done at various temperatures the 0.1 N KCl calomel electrode is better on account of the lower temperature coefficient. Since there is a variation in the value of the correction for the 0.1 N electrode used by different writers, it is hoped that some standard value may be decided on. Ostwald[8]

[4] Rosa, E. B., Vinal, G. W., and McDaniel, A. S., *Bull. of the Bureau of Standards, U. S. Dept. of Commerce*, 1913, ix, 493.

[5] Wolff, F. A., *Bull. of the Bureau of Standards, U. S. Dept. of Commerce*, 1908, v, 309.

[6] Lewis, G. N., and Randall, M., *J. Am. Chem. Soc.*, 1914, xxxvi, 1969.

[7] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

[8] Ostwald, W., and Luther, R., Physiko-chemische Messungen, Leipsic, 3rd edition. 1910.

found no difference due to size of grain of calomel, since samples
of calomel contain grains of various sizes, and the only variation
is due to impurity of ingredients or incorrect concentration of
KCl, which can easily be avoided.   It is well to seal the electrode
in a flame and have the tip of the syphon always closed by a non-
greased stop-cock or ground cap which is immersed in a closed
vessel of 0.1 N KCl solution; a second syphon connects with
saturated KCl.   Auerbach,[9] who corrects for the water vapor in
the normal hydrogen electrode, found the E.M.F. of this when
coupled with the 0.1 N KCl calomel electrode to be 0.337 at all
temperatures from 0–30°, and it would be convenient if this
value were accepted generally.   Our formula then becomes

$$P_H = - \log C_H = \frac{E.M.F. - 0.337}{0.0001984\ T}.$$ The partial pressure of hydro-

gen in the hydrogen electrode is reduced by [(760 — barometric
pressure) + (mm. CO_2) + (mm. N_2) + (vapor pressure)] and
may be made equal to 760 mm. by applying pressure to the
overflow tube of the hydrogen electrode and closing the stop-cock,

or the reading may be corrected by adding to it $\dfrac{0.0001984\ T}{2} \log q$

where q is the partial pressure of hydrogen in the electrode.
The nitrogen pressure may be taken at 10 mm. and the vapor
pressure is practically that of pure water.   The correction for
blood is about 1.7 mv. with the barometer at 740.

[9] Auerbach, F., Z. *Elektrochem.*, 1912, xviii, 13.

SOME COLOR REACTIONS FOR INDOLE AND SCATOLE.*

By V. E. NELSON.

*(From the Laboratory of Agricultural Chemistry of the University of
Wisconsin, Madison.)*

(Received for publication, February 24, 1916.)

In an effort to secure a more rapid and accurate separation and
estimation of indole and scatole in fermentation mixtures certain
substances were found to give characteristic color reactions with
one or the other of these two compounds.

Dimethylaniline when added to a solution of scatole in the
presence of sufficient sulfuric acid produces a deep red-violet
coloration which is completely soluble in chloroform. A few
drops of dimethylaniline are thoroughly emulsified with 5 or
6 cc. of scatole solution in a test-tube and concentrated sulfuric
acid is added cautiously down the side of the tube so as to form a
layer at the bottom. A beautiful violet ring forms at the junc-
tion of the two liquids which is still visible at a dilution of one
part in 1,000,000. Indole produces a faint red under similar con-
ditions, but the color is not soluble in chloroform. When sulfuric
acid is replaced by either hydrochloric or phosphoric acids no
color is developed. The sulfuric acid used was boiled so as to
free it from nitrous acid which invariably accompanies most of
the so called chemically pure acid. Dimethylaniline together
with concentrated sulfuric acid produces no color, and scatole
and sulfuric acid develop only a faint pink coloration.

Pyruvic aldehyde gives with indole, sulfuric acid, and a crystal
of ferric sulfate a deep red-violet color which cannot be extracted
by chloroform, carbon bisulfide, or amyl acetate, but is soluble
in amyl alcohol. If a few crystals of pyruvic aldehyde are added
to 5 or 6 cc. of an indole solution containing a small crystal of
ferric sulfate and sulfuric acid added cautiously so as to form a

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ment Station.

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layer at the bottom of the tube a red-violet ring forms. The test
is sensitive to about one part in 500,000. A solution of scatole
when subjected to similar treatment gives a yellow coloration
soluble in amyl acetate.

·When a few crystals of glycolic acid are dissolved in a solution
of scatole, and sulfuric acid equivalent to the volume of the solu-
tion is added, a beautiful deep red-violet color is formed. A trace
of ferric sulfate intensifies the reaction. The color is completely
soluble in chloroform, but only a slight brownish tint is extracted
by amyl acetate. Indole with glycolic and sulfuric acids gives
brown coloration which is not soluble in chloroform. The test
is easily sensitive to one part in 1,000,000.

A drop or two of a solution of glyceric aldehyde when added to
a solution of scatole in the presence of sulfuric acid produces an
intense red color completely soluble in chloroform. Indole under
similar conditions gives a yellow color insoluble in chloroform.

If to 5 or 6 cc. of an indole solution there are added a few drops
of a 5 per cent solution of vanillin in 95 per cent alcohol and the
mixture is made strongly acid with 3 or 4 cc. of concentrated hy-
drochloric acid a beautiful orange coloration forms. A solution
of scatole to which vanillin and hydrochloric acid are added gives
a deep violet color upon heating. When sulfuric acid is substi-
tuted for hydrochloric acid the colors developed by indole and
scatole are orange· and deep red to violet respectively. Phos-
phoric acid and vanillin give a deep violet with scatole and an
orange coloration with indole. If the scatole coloration is ex-
tracted with chloroform it changes to a light orange. Vanillin
and hydrobromic or acetic acids give no color with indole or sca-
tole. If hydrofluoric acid is substituted for the hydrobromic acid
then a deep violet color forms with scatole and an orange color
with indole. The violet upon extraction with chloroform changes
to a deep orange.

The exact nature of the compound formed with vanillin is not
known. It would appear from the solubility and changes of
color in chloroform that the scatole compound bears some rela-
tion to the acid used to produce it. The indole compound seems
to be the same regardless of the acid employed.

The vanillin method of detecting indole and scatole is a very
sensitive reaction. Scatole, vanillin, and hydrochloric acid give

a distinct deep violet at a dilution of one part in 2,000,000 and the color is rendered more perceptible by shaking with a few drops of chloroform. When hydrofluoric or sulfuric acids are used the test is sensitive to dilutions of one part in 2,000,000 and 3,000,000 parts respectively.

Phosphoric acid gives with vanillin and scatole a violet color up to dilutions of about one part in 2,000,000. Indole, vanillin, and hydrochloric or phosphoric acids give a test up to dilutions of one part in 5,000,000 and with sulfuric acid the color is easily discernible in a strength of one part in 2,000,000.

Steensma[1] maintained that the colors produced with indole and scatole by vanillin and hydrochloric acid could not be used as a means of their identification in a mixture of these two substances.

Because of the sensitiveness of the reaction and the distinct colors produced with these compounds and vanillin it was thought that if a solvent could be found which would extract one of the colors without extracting the other it would render the test useful qualitatively in a mixture and perhaps quantitatively also.

The following table gives the solubility in different solvents of the colors developed by indole and scatole together with vanillin, and hydrochloric, sulfuric, phosphoric, and hydrofluoric acids. Only a few of the many solvents used are given in the table.

It is to be noted that the scatole coloration is soluble in chloroform, amyl acetate, and amyl valerianate while the color produced by indole is very slightly soluble in these reagents. It is, therefore, possible to apply the test satisfactorily to a mixture of indole and scatole.

In the case of a mixture of the two compounds a few drops of a 5 per cent solution of vanillin in 95 per cent alcohol are added and sufficient acid to obtain a strong development of color. The solution is brought to a boil, cooled, and amyl acetate, amyl valerianate, or chloroform added, and shaken a few times. If hydrochloric or sulfuric acids are used the extraction of a deep violet indicates the presence of scatole. With phosphoric or hydrofluoric an orange to red color is extracted if scatole is present. With the last two acids the solution must stand for several minutes after boiling, before the maximum color develops.

[1] Steensma, F. A., Z. physiol. Chem., 1906, xlvii, 25.

TABLE I.

Solubility in Different Solvents of Colors Developed by Scatole and Indole with Vanillin and Various Acids.

Color.	Chloroform.	Amyl acetate.	Carbon disulfide.	Benzaldehyde.	Amyl valerianate.	Amyl alcohol.
Deep violet produced by boiling scatole, vanillin, and HCl.	Readily soluble.	Soluble.	Insoluble.	Soluble.	Soluble.	Soluble.
Deep red to violet produced by scatole, vanillin, and H_2SO_4.	Soluble.	"	"	"	"	Soluble but color changes to an intense blue.
Deep violet produced by boiling scatole, vanillin, and H_3PO_4.	Soluble but color changes to an orange.	Soluble but color changes to red.	"	"	Sparingly soluble with change of color to orange.	Soluble.
Deep violet produced by scatole, vanillin, and HF, on boiling and allowing to stand a few minutes.	Soluble but color changes to a deep orange.	—	—	—	—	—
Orange color produced by indole, vanillin, and HCl.	Very slightly soluble.	Insoluble.	Insoluble.	Soluble.	Insoluble.	Soluble.
Orange produced by indole, vanillin, and H_2SO_4.	Insoluble.	"	"	"	"	"
Orange developed by indole, vanillin, and H_3PO_4.	"	"	"	"	"	"

As amyl acetate, amyl valerianate, and even chloroform effect such a good separation of the colors produced by scatole and indole with vanillin and hydrochloric acid it was thought that this method might be applied quantitatively. A large number of determinations using known amounts of scatole were made and the majority of the results agreed very closely, but now and then for

some reason a large divergence was noted so that further work upon this will have to be done before the method can be pronounced satisfactory.

It might be mentioned that tryptophane also gives a coloration varying from a deep red to a deep violet with vanillin and sulfuric acid. The color is not soluble in chloroform and the reaction is not nearly so sensitive as with indole and scatole.

If to a little casein in a test-tube are added a little water, a few drops of a 5 per cent solution of vanillin in 95 per cent alcohol, and sulfuric acid, a deep violet color forms. Gelatin under similar conditions does not give this coloration.

When amyl alcohol was used to extract the color produced by scatole, vanillin, and sulfuric acid, it was noticed that the solution always becomes a distinct blue in color. It was found that amyl, butyl, and propyl alcohols together with vanillin and sulfuric acid give a very intense purple coloration which changes to a deep blue upon standing a few minutes. Methyl and ethyl alcohols do not do this. When vanillin is dissolved in amyl alcohol and concentrated HCl is added, a beautiful deep green to deep blue color develops upon heating. In propyl alcohol with the aid of hydrochloric acid and heat no color results, but after standing several minutes a light green develops. Methyl and ethyl alcohols give no color under these conditions. The compound cumarin which is so closely associated with vanillin in food analysis gives no such coloration. Phosphoric acid also gives a deep blue coloration with vanillin dissolved in amyl alcohol upon heating, but gives no color when isobutyl, propyl, ethyl, or methyl alcohols are used. When vanillin is dissolved in acetone a beautiful deep blue coloration is produced upon the addition of concentrated hydrochloric acid. Cumarin under the same conditions yields a yellow-brown color.

When xylene is added to a solution containing vanillin and concentrated hydrochloric acid a beautiful rose-red coloration goes into the acid layer upon shaking thoroughly. It would seem as though the xylene contains some substance which is able to react with the vanillin in the presence of hydrochloric acid.

SUMMARY.

1. Dimethylaniline, glycolic acid, and glyceric aldehyde give characteristic color reactions with scatole and sulfuric acid.

2. Pyruvic aldehyde gives with indole, sulfuric acid, and a crystal of ferric sulfate a deep red-violet color.

3. Vanillin produces with scatole and hydrochloric acid an intense deep violet color; with indole and hydrochloric acid, an orange coloration.

4. Vanillin gives with scatole and sulfuric acid a deep red to violet color; with indole and sulfuric acid an orange color.

5. Scatole, vanillin, and phosphoric acid give a deep violet, while indole under like conditions gives an orange coloration. When hydrofluoric acid is substituted for phosphoric acid a deep violet is produced with scatole and an orange coloration with indole.

6. From the solubility tests it appears that the compound formed with scatole, vanillin, and various acids bears some relation to the acids used to produce it. The compound with indole is the same regardless of the acid employed.

7. By using chloroform, amyl acetate, or amyl valerianate it is possible to apply the vanillin method qualitatively in the case of a mixture of indole and scatole.

8. The intensity of the color produced by scatole, vanillin, and hydrochloric acid seems to be proportional to the concentration of scatole, but further work must be done before the method can be made quantitative.

9. Vanillin in amyl, butyl, or propyl alcohols gives characteristic color reactions with hydrochloric, sulfuric, or phosphoric acids. Vanillin in acetone gives a deep blue coloration upon the addition of hydrochloric acid. A rose-red is developed when xylene, vanillin, and hydrochloric acid are thoroughly shaken together.

10. A few drops of a 5 per cent solution of vanillin in 95 per cent alcohol when added to a solution of tryptophane in the presence of sufficient sulfuric acid produce a deep red to violet coloration.

INDOLE IN CHEESE.*

By V. E. NELSON.

(*From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.*)

In attempting to isolate certain amines and other decomposition products resulting from the action of various microorganisms upon amino-acids found in cheese it was observed that limburger and *Handkäse* contain a considerable amount of indole and that the former also contains an appreciable quantity of phenolic bodies. This led to a study of other varieties of both soft and hard cheese to determine whether they contained such substances as indole, scatole, and phenol.

The method of procedure was to macerate about 400 gm. of cheese with water, place the mixture in a 5 liter flask, and distil with steam. The distillate might contain indole, scatole, phenol, and ammonia. The phenol was separated from the remaining substances by making the distillate alkaline with NaOH and redistilling. The ammonia was removed by rendering the last distillate acid with dilute sulfuric acid and redistilling. The tests applied for the detection of indole were: Herter's β-naphthaquinone reaction, Konto's test, cholera red reaction, Legal's reaction, pine wood test, pyruvic aldehyde reaction, nitroso-indole nitrate, and vanillin tests. For scatole the following tests were employed —the dimethylaniline test, Herter's p-dimethylamino benzaldehyde reaction, and the glycolic acid and vanillin tests. For the detection of phenol, Millon's reagent was used.

It will be observed in the table that of all the different cheeses examined only three varieties, namely, limburger, *Handkäse*, and camembert, contain any of these putrefactive substances. Furthermore, no scatole accompanies the indole and phenol. What-

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ever organism in cheese is concerned in the transformation of tryptophane into indole does so without the production of scatole. Camembert cheese contains considerably less indole than limburger or *Handkäse*. In fact in the cheese examined which was imported from Germany not more than traces could be detected. No scatole or phenol was present in this type of cheese.

Cheese.	Indole.	Scatole.	Phenol.
Limburger........................	Present.	None.	Present.
Handkäse........................	"	"	None.
Cheddar........................	None.	"	"
Swiss........................	"	"	"
Gammalost........................	"	"	"
Brick........................	"	"	"
Roquefort........................	"	"	"
Camembert........................	Trace.	"	"

Fifteen different limburger cheeses were examined and in no case was indole found to be absent. In a few very young samples which had not ripened sufficiently it was present in very small amounts. Scatole was always absent. The *Handkäse* used was not sufficiently ripe to say absolutely that no scatole or phenol occur in this type of cheese. The two samples examined gave distinct tests for indole, but not for scatole or phenol. We were unable to obtain further samples because of the scarcity of the cheese in this vicinity.

In order to get some idea as to the amount of indole found in a good ripe limburger cheese quantitative estimations were made according to Herter's method[1] as follows:

A 100 gm. sample of limburger cheese was macerated with water and after rendering the mixture alkaline with potassium hydroxide it was steam distilled. The distillate contained ammonia and indole, the phenol being held back by the caustic potash. The ammonia was removed by redistilling from a dilute sulfuric acid solution. The distillation was continued until no coloration was given by vanillin and sulfuric acid. To the distillate made alkaline with sodium hydroxide was added a slight excess of a 2 per cent solution of sodium naphthaquinone monosulfonate and

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[1] Herter, C. A., and Foster, M. L., *J. Biol. Chem.*, 1905–06, i. 257.

the solution was allowed to stand for at least 10 minutes. The blue color which developed was shaken out completely by chloroform and the color compared in a Duboscq colorimeter with a known amount of indole dissolved in water and treated precisely in the same manner. The amount of indole found in various cheeses depended upon how far the ripening process had proceeded. Some of the young cheese contained such small amounts of indole that it was impossible to determine it quantitatively. Others contained indole to the extent of one part in 500,000 while the highest amount found was one part of indole in 52,800 parts of cheese.

Work was begun to determine what organisms are responsible for the production of indole in limburger cheese. Culture media containing tryptophane were inoculated with the three main types of organisms found in cheese; namely, the lactic, bulgaricus, and coccus forms. The composition of the medium was as follows:

	gm.
Tryptophane	0.1176
Lactose	4.0
K_2HPO_4	0.3
$MgSO_4.7H_2O$	0.1
NaCl	0.1
$CaCO_3$	2.0
$FeSO_4$	Trace.
Tap water	1,000 cc.

After standing for several months the flasks were opened and examined for indole, scatole, and phenol. None of these substances could be detected in the lactic and bulgaricus cultures. In the case of the coccus organism traces of indole were formed. This organism which had considerable liquefying power was isolated from a cheddar cheese and the surprising thing is that no indole is found in this type of cheese. Only one of the media was inoculated with the coccus organism. If the experiments which are now in progress prove that indole is formed by this type of organism then the explanation why no indole is found in cheddar cheese may be that the conditions prevailing are unfavorable for the growth of this organism. It is possible that the growth of the lactic and bulgaricus organisms is not vigorous enough in this kind of a medium to effectively attack the amino-acid. To obviate this it may be necessary to add to the culture a little peptone

or milk, which ought to increase the activity of the organisms without impairing the results. Until this has been done it will be impossible to say that the lactic and bulgaricus organisms do not produce these putrefactive substances. Work along this line is now in progress and will be reported later.

SUMMARY.

1. Indole and phenol were found to be present in limburger cheese.

2. Scatole was not found in limburger cheese.

3. Indole is present in *Handkäse*. It is doubtful if scatole and phenol are to be found in this type of cheese.

4. A trace of indole is present in camembert cheese. Scatole and phenol are absent in this type of cheese.

5. Cheddar, Swiss, *Gammalost*, brick, and roquefort do not contain any indole, scatole, or phenolic bodies.

6. The amount of indole in a limburger cheese naturally varies, depending upon how far the ripening process has gone. A young cheese may contain such a small amount that a quantitative estimation is impossible while a good ripe limburger cheese may contain as much as one part in 52,800 parts of cheese.

7. Lactic and bulgaricus forms of organisms when grown upon media containing tryptophane produced no indole or scatole. As growing organisms upon pure amino-acids is more difficult than upon proteins it may be necessary to add a little peptone or milk to the culture and until further work has been done upon this phase it will be impossible to say that these organisms do not produce these putrefactive substances.

8. The liquefying coccus isolated from a cheddar cheese appears to produce traces of indole from tryptophane. If the experiments now in progress confirm this statement, then the explanation why no indole is produced by this type of organism in cheddar cheese must be that conditions are unfavorable and growth is suppressed.

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